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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/GB98/02851</p> <p>(22) International Filing Date: 21 September 1998 (21.09.98)</p> <p>(30) Priority Data: 9720054.7 19 September 1997 (19.09.97) GB</p> <p>(71) Applicant (<i>for all designated States except US</i>): CELLTECH THERAPEUTICS LIMITED [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB).</p> <p>(72) Inventor; and</p> <p>(75) Inventor/Applicant (<i>for US only</i>): HUMPHREYS, David, Paul [GB/GB]; 76 Stonefield Park, Maidenhead, Berkshire SL6 6ES (GB).</p> <p>(74) Agent: MERCER, Christopher, Paul; Carpmaels &amp; Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report.</i></p> <p>(88) Date of publication of the international search report: 17 June 1999 (17.06.99)</p>	
<p>(54) Title: PEPTIDE SEQUENCES AS HINGE REGIONS IN PROTEINS LIKE IMMUNOGLOBULIN FRAGMENTS AND THEIR USE IN MEDICINE</p> <p>(57) Abstract</p> <p>Peptides of the formula (1) <math>^N\text{TCPPCPXYCPPCPA}^C</math> are described wherein X and Y, which may be the same or different, is each a neutral aliphatic L-amino acid residue, and protected and reactive derivatives thereof. The peptides may be used as hinge regions in proteins, where they are capable of being covalently coupled to achieve dimeric structure, for example as found in antibodies.</p>			

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# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/GB 98/02851

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C07K16/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>M.L. RODRIGUES ET AL.: "Engineering Fab' Fragments for Efficient F(ab)2 Formation in Escherichia coli and for Imrpobed In Vivo Stability"          JOURNAL OF IMMUNOLOGY,          vol. 151, no. 12, 15 December 1993, pages          6954-6961, XP002098809          BALTIMORE US          cited in the application          see page 6959, right-hand column,          paragraph 2</p> <p>---</p> <p>-/-</p>	1-8

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the International search

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# INTERNATIONAL SEARCH REPORT

In International Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HUMPHREYS, DAVID P. ET AL: "Formation of dimeric Fab's in Escherichia coli: effect of hinge size and isotype, presence of interchain disulfide bond, Fab' expression levels, tail piece sequences and growth conditions" J. IMMUNOL. METHODS (1997), 209(2), 193-202 CODEN: JIMMBG; ISSN: 0022-1759, 1 December 1997, XP004103797 see page 198, left-hand column, paragraph 2 - right-hand column, paragraph 2; table 1  ---	1-8
A	M. BETTER ET AL.: "Potent anti-CD5 ricin A chain immunoconjugates from bacterially produced Fab' and F(ab')2" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 2, 15 January 1993, pages 457-461, XP002098811 WASHINGTON US cited in the application see figure 1  ---	1,7
A	EP 0 284 898 A (MAX PLANCK GESELLSCHAFT) 5 October 1988 see claims; examples -----	1-8

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

Int'l Application No

PCT/GB 98/02851

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 0284898 A	05-10-1988	JP 63258895 A	26-10-1988	US 5041533 A 20-08-1991

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant (for all designated States except US): CELLTECH THERAPEUTICS LIMITED [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB).  (72) Inventor; and (75) Inventor/Applicant (for US only): HUMPHREYS, David, Paul [GB/GB]; 76 Stonefield Park, Maidenhead, Berkshire SL6 6ES (GB).  (74) Agent: MERCER, Christopher, Paul; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).			
(54) Title: PEPTIDES			
(57) Abstract <p>Peptides of the formula (1) <math>N\text{TCPPCPXYCPPCPAC}</math> are described wherein X and Y, which may be the same or different, is each a neutral aliphatic L-amino acid residue, and protected and reactive derivatives thereof. The peptides may be used as hinge regions in proteins, where they are capable of being covalently coupled to achieve dimeric structure, for example as found in antibodies.</p> <p style="text-align: right;"><b><math>N\text{TCPPCPXYCPPCPAC}</math> (1)</b></p>			

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**PEPTIDES**

This invention relates to peptides which function as hinge regions in proteins, to proteins containing such hinge regions and to the use of said proteins in medicine.

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In clinical antibody therapy and imaging applications the avidity of a dimeric antibody species is often required to achieve an effective antibody affinity *in vivo*, but without the effector functions or lengthy serum permanence conferred by the Fc domain (ref. 1-3 – for the literature referenced by number herein see the list "References" 10 hereinafter). F(ab')<sub>2</sub> molecules meet this requirement and can be produced by proteolytic cleavage of monoclonal IgG of appropriate isotypes, or by use of recombinant immunoglobulin derived domains produced in *E.coli*.

The ability to secrete antibody fragments to the oxidising periplasm of *E.coli* has led 15 to rapid advances in the engineering of grafted, highly expressed Fab's. Importantly, the use of *E.coli* also enables the cost effective and rapid production of the amounts of antibody material required to supply a large market (ref. 4, 5). Engineered Fab' is often expressed with only one hinge-cysteine. This cysteine can be used for attachment of other Fab's to make a F(ab')<sub>2</sub> or attachment of therapeutic effector 20 molecules such as radionuclides, enzymes, or toxins (ref. 6).

Several protein engineering approaches for producing divalent antigen binding species *in vivo* in *E.coli* have been reported, using both modified scFvs and Fab's. Simple hinge modifications do not give substantial yields *in vivo* of dimeric species 25 from *E.coli* (ref. 7-9). Techniques for increasing dimerisation *in vivo* are well characterised, but these often use large non-immunoglobulin dimerisation motifs which are potentially immunogenic and can cause severe reductions in the level of expression of soluble protein (ref. 10-13). The simplest route to production of dimeric antigen binding species remains the direct disulphide or chemical cross-linking of 30 Fab's *in vitro* (ref. 2, 7, 14, 15). The choice of covalent linkage between the two Fab's is an important one. If the F(ab')<sub>2</sub> is cleaved *in vivo* then the resulting Fab' molecules generated suffer both from loss of avidity and very rapid clearance from the circulation (ref. 1, 2). Single disulphide bonds are known to be more susceptible 35 to cleavage *in vivo* than protected disulphides, sulphide, or thioether bonds (ref. 2, 9, 16). However, two disulphides as found in the hinge region of F(ab')<sub>2</sub> isolated from proteolytic cleavage of IgG1 have previously been found to be as robust as one thioether bond, as judged by serum permanence (ref. 9).

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There is a need for non-immunogenic dimeric antibody species which overcome the problem of facile *in vivo* cleavage while still being efficient to manufacture and couple to other effector molecules. We have now found a peptide, which when part of a larger protein such as a Fab' fragment efficiently generates dimers and yields dimeric  
5 material which is highly resistant to chemical reduction *in vitro* and has long serum permanence times *in vivo*. Advantageously, the peptide is well tolerated in *E.coli* and in our tests to date has been shown to be non-immunogenic.

Thus according to one aspect of the invention we provide a peptide of formula (1):

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wherein X and Y, which may be the same or different, is each a neutral aliphatic L-amino acid residue, and protected and reactive derivatives thereof.

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In formula (1) and the other peptides described herein conventional single letter abbreviations are used to represent amino acid residues except where otherwise indicated. The superscripts "N" or "C" are used to indicate respectively the N- or C-terminal residue of a peptide.

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Neutral L-amino acid residues represented by each of the groups X and Y include glycine, alanine, valine, leucine, isoleucine, serine and threonine residues.

25

Protected derivatives of the peptides of formula (1) include N- and C-terminal protected derivatives in which the N-terminal amino group or the C-terminal carboxyl group is linked to a protecting group. N-protected derivatives include for example optionally substituted benzyloxy-carbonylamino, allyloxycarbonylamino, cycloalkyloxycarbonylamino, t-butoxycarbonylamino, trifluoroacetylarnino, phthalylarnino, aralkylarnino, e.g. benzylarnino, diphenylmethylnino or triphenylmethylnino, tosyl-arnino or formylarnino derivatives. C-protected derivatives include for example esters, such as optionally substituted alkyl, e.g. methyl, ethyl or t-butyl, aralkyl, e.g. benzyl or benzhydryl, silyl, e.g. trimethylsilyl and phthalimidomethyl esters, and esters with polymers, for example functionalised styrene-based resins.

35

Reactive derivatives of peptides of formula (1) include those in which the C-terminal carboxyl group is functionalised, and is, for example, an acyl halide such as an acyl chloride, an acyl cyanide or azide, an anhydride, an ester, for example a N-hydroxy

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succinimide, p-nitrophenyl or pentachloro-phenyl ester, a N-acyl heterocycle such as a N-acyl imidazole, pyrazole or triazole or an activated acid formed by addition of a carbodiimide or isoxazolium reagent.

- 5 Particularly useful peptides of formula (1) include those wherein X is an alanine residue. In another preference, Y in particular is a threonine residue. An especially useful peptide according to the invention has the formula (2):



10

and protected and reactive derivatives thereof.

The peptides according to the invention may be prepared from appropriately activated and/or protected amino acids [for example utilising reactive derivatives and 15 protecting groups of the types mentioned in connection with the peptides of formula (1)] using routine peptide synthesis techniques [see for example Merrifield, B. Science (1985), 232, 341-347].

20 The presence of four cysteine residues in each peptide of formula (1) provides four centres for disulphide and/or thioether bond formation thus allowing reaction with other molecules containing thiol reactive groups. In particular dimeric proteins may be obtained by incorporating the peptides in protein chains and the invention extends to such a use. In order to achieve protein dimerisation a peptide of formula (1) must first be coupled to an existing protein chain or synthesised *de novo* as part of it and 25 we therefore provide in another aspect of the invention a protein comprising one polypeptide chain characterised in that said chain contains an amino acid sequence  $\text{NTCPPCPXYCPCPAC}$ , wherein X and Y are as defined for the peptide of formula (1).

30 The invention also extends to proteins containing two polypeptide chains covalently linked via the cysteine residues in  $\text{NTCPPCPXYCPCPAC}$  as explained above and thus according to a further aspect of the invention we provide a protein comprising two polypeptide chains characterised in that each of said chains contains an amino acid sequence  $\text{NTCPPCPXYCPCPAC}$  [wherein X and Y are as defined for the peptide of formula (1)] and each chain is covalently linked to the other through one, two, three or four of the cysteine residues present in each of said amino acid sequences.

In the proteins according to the invention the amino acid sequence NTCPPCPXYPCCPAC is preferably a sequence NTCPPCPAYCPCPAC or NTCPPCPXTCPCCPAC, especially the sequence NTCPPCPATCPCPAC.

- 5 The proteins according to the invention may be naturally occurring proteins to which the peptide of formula (1) has been coupled, or recombinant proteins incorporating the amino acid sequence NTCPPCPXYPCCPAC. The proteins may generally be structural or, especially, binding proteins. Particular binding proteins include hormones, cytokines, colony stimulating factors, growth factors, releasing factors, ion carriers, toxins, and receptors thereof, including all or part of receptors associated with binding to cell surface associated molecules, the T-cell receptor, CD4, CD8, CD28, cytokine receptors, e.g. an interleukin receptor, TNF receptor or interferon receptor, receptors for colony stimulating factors e.g. G-CSF or GM-CSF, platelet derived growth factors e.g. PDGF- $\alpha$  and PDGF- $\beta$ , and in particular antibodies and antigen binding fragments thereof.
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- 15

Where the protein according to the invention has one peptide chain containing a NTCPPCPXYPCCPAC amino acid sequence it may be a monomeric protein or be separately linked to one or more other polypeptide chains to form overall a multimeric structure. In those proteins of the invention containing two polypeptide chains with covalently linked NTCPPCPXYPCCPAC sequences the protein will clearly be at least dimeric but may also consist of other, separately linked chains to form overall a multimeric structure. Dimers and multimers may be composed of more than one type of polypeptide chain and may be homo- or heteromeric.

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  - 35
- The production of proteins according to the invention may be achieved using standard chemical or recombinant DNA techniques. Chemical techniques include chemical coupling of a protein and a peptide of formula (1) using activated and protected derivatives of the protein and peptide as described above in relation to the production of peptides of formula (1). In this way, for example, a peptide of formula (1) may be site-specifically coupled to the C-terminal end of a suitably C-activated protein.

Recombinant DNA techniques generally involve the expression of a protein by a host cell, followed by recovery of the protein using standard separation and purification techniques. DNA coding for the protein may be introduced into any suitable expression vector by operatively linking the DNA to any necessary expression control elements therein and transforming any suitable prokaryotic or eucaryotic host cell

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with the vector using well known procedures. A more detailed description of suitable techniques is given hereinafter in relation to the production of antibodies according to the invention. These may be generally followed and/or easily adapted to enable the production of any protein according to the invention by recombinant means. The use  
5 of recombinant DNA technology provides a flexible approach to proteins according to the invention in that it enables the easy manipulation and insertion of a peptide of formula (1) at any desired position in a protein. DNA coding for a peptide of formula (1) is thus particularly useful and forms a further aspect of the invention. DNA containing the following nucleotide sequence:

10 5' ACATGCCCGCCGTGCCCGGCGACCTGCCCGCCGTGCCCGGCG 3'  
[where each letter is the standard code for a nucleotide] coding for the amino acid sequence  $^N\text{TCPPCPATCPPCAC}$  is especially useful and the invention extends to DNA comprising this sequence together with variants thereof wherein one or more  
15 nucleotides have been substituted due to the degeneracy of the genetic code. The invention also extends to recombinant plasmids containing DNA coding for a peptide of formula (1), to cells containing said plasmids and to a process for producing a protein according to the invention which comprises culturing said cells such that the desired protein is expressed and recovering the protein from the culture

20 25 The amino acid sequences  $^N\text{TCPPCPXYCPPCPAC}$  are particularly suitable for use with binding proteins such as cell-associated receptors or antibodies where each sequence can function as a hinge region to provide a dimerisation capacity. Thus, for example, recombinant receptors [see for example International Patent Specifications Nos. WO 92/100591, WO 92/15322, WO 93/19163, WO 95/02686 and WO 97/23613] can be designed which incorporate these sequences to facilitate dimerisation of the individual receptor chains required for efficient receptor binding and the invention extends to a recombinant receptor comprising at least one peptide of formula (1). Such receptors are of use for example to redirect and activate cells  
30 [see the patent specifications just mentioned] and the invention includes such cells expressing a recombinant receptor comprising at least one peptide of formula (1).

35 In antibodies the sequences  $^N\text{TCPPCPXYCPPCPAC}$  can be used as substitutes for naturally occurring hinge regions [the hinge region is located between the  $\text{C}_\text{H}1$  and  $\text{C}_\text{H}2$  domains in naturally occurring immunoglobulins]. The sequences are particularly suited for this purpose and in a preferred aspect of the invention we provide an antibody containing a hinge region characterised in that said hinge region has an amino acid sequence  $^N\text{TCPPCPXYCPPCPAC}$  where X and

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Y are as defined for formula (1). In this instance, the hinge region preferably has an amino acid sequence NTCPPCPAYCPCPAC or NTCPPCPXTCPCPAC, more especially NTCPPCATCPCPAC.

- 5 The term "antibody" as used herein is generally intended to include monovalent, divalent or other multivalent antibodies. Thus for example a monovalent antibody according to the invention may be a single chain comprising an immunoglobulin heavy chain variable ( $V_H$ ) domain and a NTCPPCPXYCPCPAC hinge region directly attached to it, preferably at the C-terminal end of the  $V_H$  domain.
- 10 Alternatively, the  $V_H$  domain and hinge region may be separated by a spacer region comprising one or more amino acids in peptide linkage to each other and the rest of the antibody. The spacer region may be for example one or more immunoglobulin heavy ( $C_H$ ) or light ( $C_L$ ) chain constant domains or fragments thereof for example a  $C_H1$  domain or a fragment thereof and/or a  $C_H2$  and/or a  $C_H3$  domain or fragments
- 15 thereof. Where desired the hinge region may have one or more other amino acids attached to its C-terminus for example one or more immunoglobulin constant region domains or fragments thereof as just described. The  $V_H$  domain may be monomeric or it may be dimeric and contain  $V_H$ - $V_H$  or  $V_H$ - $V_L$  (where  $V_L$  is an immunoglobulin light chain variable domain) dimers in which the  $V_H$  and  $V_L$  chains are non-covalently
- 20 associated. Where desired however, the chains may be covalently coupled either directly, for example via a disulphide bond between the two variable domains, or through a linker, for example a peptide linker, to form a single chain domain. Particular examples of monovalent antibodies according to the invention include Fv, single chain Fv and especially Fab or Fab' fragments each containing a hinge region
- 25 having the sequence NTCPPCPXYCPCPAC.

Divalent antibodies according to the invention include two of the monomeric chains just described covalently linked through one, two, three or four of the cysteine residues of the hinge region NTCPPCPXYCPCPAC of each chain. The linkage may be a simple disulphide linkage or may be via a linker group, for example as described in International Patent Specifications Nos. WO 90/09195 and WO 90/09196. Particular divalent antibodies include  $F(ab)_2$  and  $F(ab')_2$  fragments.

Multivalent antibodies according to the invention include for example tri- and tetravalent antibodies comprising three or four of the monomeric chains described above linked via their hinge regions by a linker, for example as described in International Patent Specification No. 92/22583.

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The antibody according to the invention will in general be capable of selectively binding to an antigen. The antigen may be any cell-associated antigen, for example a cell surface antigen such as a T-cell, endothelial cell or tumour cell marker, or it may be a soluble antigen. Particular examples of cell surface antigens include

- 5 adhesion molecules, such as E-selectin, P-selectin or L-selectin, CD2, CD3, CD4, CD5, CD7, CD8, CD11a, CD11b, CD18, CD19, CD20, CD23, CD25, CD33, CD38, CD40, CD45, CDW52, CD69, carcinoembryonic antigen (CEA), human milk fat globulin (HMFG1 and 2), MHC Class I and MHC Class II antigens, and VEGF, and where appropriate, receptors thereof. Soluble antigens include interleukins such as
- 10 IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8 or IL-12, viral antigens, for example respiratory syncytial virus or cytomegalovirus antigens, interferons such as interferon- $\alpha$ , interferon- $\beta$  or interferon- $\gamma$ , tumour necrosis factor- $\alpha$ , tumour necrosis factor- $\beta$ , colony stimulating factors such as G-CSF or GM-CSF, and platelet derived growth factors such as PDGF- $\alpha$ , and PDGF- $\beta$  and where appropriate receptors thereof.

- 15
- The variable region domain(s) may be any naturally occurring variable domain or an engineered version thereof. By engineered version is meant a variable region domain which has been created using recombinant DNA engineering techniques. Such engineered versions include those created for example from natural antibody
- 20 variable regions by insertions, deletions or changes in or to the amino acid sequences of the natural antibodies. Particular examples of this type include those engineered variable region domains containing at least one complementarity determining region (CDR) and optionally one or more framework amino acids from one antibody and the remainder of the variable region domain from a second
- 25 antibody.

- Antibodies according to the invention may be obtained from any whole antibody, especially a whole monoclonal antibody, [prepared by conventional immunisation and cell fusion procedures], using any suitable standard enzymatic cleavage and/or digestion techniques, for example by treatment with pepsin followed by chemical coupling with a peptide of formula (1) or a protected or activated derivative thereof using routine protein synthesis techniques as described above in relation to the production of peptides of formula (1). Alternatively, the antibody of the invention may be prepared by the use of recombinant DNA techniques involving the manipulation and re-expression of DNA encoding antibody variable and/or constant regions. Such DNA is known and/or is readily available from DNA libraries including for example phage-antibody libraries [see Chiswell, D. J. and McCafferty, J. Tibtech. 10 80-84 (1992)] or where desired can be synthesised. Standard molecular biology and/or

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chemistry procedures may be used to sequence and manipulate the DNA, for example, to introduce codons to create a sequence NTCPPCPXYCPCPAC, to modify, add or delete other amino acids or domains as desired.

- 5 From here, one or more replicable expression vectors containing the DNA may be prepared and used to transform an appropriate cell line, e.g. a non-producing myeloma cell line, such as a mouse NSO line or a bacterial, e.g. *E.coli* line, in which production of the antibody will occur. In order to obtain efficient transcription and translation, the DNA sequence in each vector should include appropriate regulatory  
10 sequences, particularly a promoter and leader sequence operably linked to the variable domain sequence. Particular methods for producing antibodies in this way are generally well known and routinely used. For example, basic molecular biology procedures are described by Maniatis *et al* [Molecular Cloning, Cold Spring Harbor Laboratory, New York, 1989]; DNA sequencing can be performed as described in  
15 Sanger *et al* [PNAS 74, 5463, (1977)] and the Amersham International plc sequencing handbook; and site directed mutagenesis can be carried out according to the method of Kramer *et al* [Nucl. Acids Res. 12, 9441, (1984)] and the Anglian Biotechnology Ltd handbook. Additionally, there are numerous publications, including patent specifications, detailing techniques suitable for the preparation of  
20 antibodies by manipulation of DNA, creation of expression vectors and transformation of appropriate cells, for example as reviewed by Mountain A and Adair, J R in Biotechnology and Genetic Engineering Reviews [ed. Tombs, M P, 10, Chapter 1, 1992, Intercept, Andover, UK] and in International Patent Specification No. WO 91/09967. Once expressed, the antibody may be separated from the host  
25 cell and purified using standard centrifugation, filtration, chromatography and other separation/purification techniques, for example as described in the Examples hereinafter.

30 Antibody fragments containing a sequence NTCPPCPXYCPCPAC, particularly Fab or Fab' fragments are particularly suited for manufacture in *E.coli* as described above and in the Examples herein.

Where desired, the protein, including antibody, according to the invention may have one or more effector or reporter molecules attached to it and the invention extends to  
35 such modified proteins and, in particular, antibodies. The effector or reporter molecules may be attached to the protein through any available amino acid side-chain or terminal amino acid functional group located in the protein, for example any free amino, imino, hydroxyl or carboxyl group. In one preference however the

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molecule may be attached to a cysteine residue in an amino acid sequence  $\text{N}(\text{CPCPXYCP})\text{CPAC}$ . Dimers containing these sequences are particularly resistant to chemical reduction *in vitro* and advantageously can be partially reduced to expose reactive thiols to which effector or reporter molecules may be attached.

- 5 One, two, three or more effector or reporter molecules may be attached in this way.

Effector molecules include, for example, antineoplastic agents, toxins (such as enzymatically active toxins of bacterial or plant origin and fragments thereof e.g. ricin and fragments thereof) biologically active proteins, for example enzymes, synthetic or

- 10 naturally occurring polymers, nucleic acids and fragments thereof, e.g. DNA, RNA and fragments thereof, radionuclides, particularly radioiodide, and chelated metals. Suitable reporter groups include chelated metals, fluorescent compounds or compounds which may be detected by NMR or ESR spectroscopy.

- 15 Particular antineoplastic agents include cytotoxic and cytostatic agents, for example alkylating agents, such as nitrogen mustards (e.g. chlorambucil, melphalan, mechlorethamine, cyclophosphamide, or uracil mustard) and derivatives thereof, triethylenephosphoramide, triethylenethiophosphor-amide, busulphan, or cisplatin; antimetabolites, such as methotrexate, fluorouracil, floxuridine, cytarabine, mercaptopurine, thioguanine, fluoroacetic acid or fluorocitric acid, antibiotics, such as bleomycins (e.g. bleomycin sulphate), doxorubicin, daunorubicin, mitomycins (e.g. mitomycin C), actinomycins (e.g. dactinomycin) plicamycin, calicheamicin and derivatives thereof, or esperamicin and derivatives thereof; mitotic inhibitors, such as etoposide, vincristine or vinblastine and derivatives thereof; alkaloids, such as ellipticine; polyols such as taxicin-I or taxicin-II; hormones, such as androgens (e.g. dromostanolone or testolactone), progestins (e.g. megestrol acetate or medroxyprogesterone acetate), estrogens (e.g. dimethylstilbestrol diphosphate, polyestradiol phosphate or estramustine phosphate) or antiestrogens (e.g. tamoxifen); anthraquinones, such as mitoxantrone, ureas, such as hydroxyurea; hydrazines, such as procarbazine; or imidazoles, such as dacarbazine.

Synthetic or naturally occurring polymers include for example optionally substituted straight or branched chain polyalkylene, polyalkenylene or polyoxyalkylene polymers such as polyethyleneglycol, polypropylene glycol, polyvinylalcohol and especially, 35 methoxypolyethylene glycol or branched or unbranched polysaccharides, e.g. a homo- or hetero-polysaccharide such as lactose, amylose, dextran or glycogen.

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Chelated metals include chelates of di-or tripositive metals having a coordination number from 2 to 8 inclusive. Particular examples of such metals include technetium (Tc), rhenium (Re), cobalt (Co), copper (Cu), gold (Au), silver (Ag), lead (Pb), bismuth (Bi), indium (In), gallium (Ga), yttrium (Y), terbium (Tb), gadolinium (Gd), and 5 scandium (Sc). In general the metal is preferably a radionuclide. Particular radionuclides include  $^{99m}\text{Tc}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{58}\text{Co}$ ,  $^{60}\text{Co}$ ,  $^{67}\text{Cu}$ ,  $^{195}\text{Au}$ ,  $^{199}\text{Au}$ ,  $^{110}\text{Ag}$ ,  $^{203}\text{Pb}$ ,  $^{206}\text{Bi}$ ,  $^{207}\text{Bi}$ ,  $^{111}\text{In}$ ,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{88}\text{Y}$ ,  $^{90}\text{Y}$ ,  $^{160}\text{Tb}$ ,  $^{153}\text{Gd}$  and  $^{47}\text{Sc}$ .

10 The chelated metal may be for example one of the above types of metal chelated with any suitable polydentate chelating agent, for example acyclic or cyclic polyamines, polyethers, (e.g. crown ethers and derivatives thereof); polyamides; porphyrins; and carbocyclic derivatives.

15 In general, the type of chelating agent will depend on the metal in use. One particularly useful group of chelating agents in conjugates according to the invention, however, are acyclic and cyclic polyamines, especially polyaminocarboxylic acids, for example diethylenetriaminepentaacetic acid and derivatives thereof, and macrocyclic amines, e.g. cyclic tri-aza and tetra-aza derivatives (for example as described in International Patent Specification No. WO 92/22583); and polyamides, especially 20 desferriox-amine and derivatives thereof.

Particularly useful effector groups are calichaeemicin and derivatives thereof (see for example South African Patent Specifications Nos. 85/8794, 88/8127 and 90/2839).

25 Where it is desired to obtain a protein according to the invention linked to an effector or reporter molecule this may be prepared by standard chemical or recombinant DNA procedures in which the protein is linked either directly or via a coupling agent to the effector or reporter molecule. Particular chemical procedures include for example those described in International Patent Specification Nos. WO 93/06231, WO 30 92/22583, WO 90/09195 and WO 89/01476 and the Examples herein utilising functional groups e.g. thiols in the protein and where necessary appropriately activated effector or reporter molecules, for example thiol selective derivatives such as maleimides where the target is a protein thiol group. Alternatively, where the effector or reporter molecule is a protein or polypeptide the linkage may be achieved 35 using recombinant DNA procedures, for example as described herein or in International Patent Specification No. WO 86/01533 and European Patent Specification No. 392745.

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The protein according to the invention may be useful in the detection or treatment of a number of diseases or disorders. Such diseases or disorders may include those described under the general headings of infectious disease, e.g. viral infection; inflammatory disease/autoimmunity e.g. rheumatoid arthritis, osteoarthritis,

- 5 inflammatory bowel disease; cancer; allergic/atopic disease e.g. asthma, eczema; congenital disease, e.g. cystic fibrosis, sickle cell anaemia; dermatologic disease, e.g. psoriasis; neurologic disease, e.g. multiple sclerosis; transplants e.g. organ transplant rejection, graft-versus-host disease; and metabolic/ idiopathic disease e.g., diabetes.

10

The proteins according to the invention may be formulated for use in therapy and/or diagnosis and according to a further aspect of the invention we provide a pharmaceutical composition comprising a protein according to the invention together with a pharmaceutically acceptable excipient, diluent or carrier. As explained above, 15 the protein in this aspect of the invention may be optionally linked to one or more effector or reporter groups.

- 20 The pharmaceutical composition may take any suitable form for administration, and, preferably is in a form suitable for parenteral administration e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the composition is for injection or infusion, it may take the form of a suspension, solution or emulsion in an oily or aqueous vehicle and it may contain formulatory agents such as suspending, preservative, stabilising and/or dispersing agents.

- 25 Alternatively, the protein composition may be in dry form, for reconstitution before use with an appropriate sterile liquid.

- If the protein composition is suitable for oral administration the formulation may contain, in addition to the active ingredient, additives such as starch, e.g. potato, 30 maize or wheat starch, or cellulose or starch derivatives such as microcrystalline cellulose; silica; various sugars such as lactose; magnesium carbonate and/or calcium phosphate. It is desirable that, if the formulation is for oral administration it will be well tolerated by the patient's digestive system. To this end, it may be desirable to include in the formulation mucus formers and resins. It may also be 35 desirable to improve tolerance by formulating the protein in a capsule which is insoluble in the gastric juices. It may also be preferable to include the protein or composition in a controlled release formulation.

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If the protein composition is suitable for rectal administration the formulation may contain a binding and/or lubricating agent; for example polymeric glycols, gelatins, cocoa-butter or other vegetable waxes or fats.

- 5 Therapeutic and diagnostic uses of proteins according to the invention typically comprise administering an effective amount of the protein to a human subject. The exact amount to be administered will vary according to the use of the protein and on the age, sex and condition of the patient but may typically be varied from about 0.1mg to 1000mg for example from about 1mg to 500mg. The protein may be  
10 administered as a single dose or in a continuous manner over a period of time. Doses may be repeated as appropriate. Typical doses may be for example between 0.1-50mg/kg body weight per single therapeutic dose, particularly between 0.1-20 mg/kg body weight for a single therapeutic dose.
- 15 The following Examples illustrate the invention. The following abbreviations are used:  
AUC area under curve;                    $\beta$ -ME  $\beta$ -mercaptoethylamine;  
DCM Di-Fab' Maleimide;               DTDP 4,4'dithiodipyridine;  
Fab' antigen binding antibody fragment (with hinge);  
20 F(ab')<sub>2</sub> dimeric Fab';              HC heavy chain;            LC light chain;  
NEM N-ethylmaleimide;                PEG polyethylene glycol.

**EXAMPLE 1**

**Evaluation of di-Fab' Production in *E.coli* utilising different hinge sequences.**

- 25 Strategy - To minimise any possible incorrect interchain disulphide bonds between hinge regions and any other cysteines the interchain disulphide bond was removed from all Fab' constructs. It has been shown previously that removal of the interchain disulphide bond of a di-Fab' did not affect the stability of the protein as judged by serum permanence times (ref. 9). PCR mutagenesis was used to change the interchain cysteines of cKappa and C<sub>H</sub>1 to serines. This also enabled analysis of %  
30 di-Fab' formation by analysing periplasmic extracts on non-reducing SDS-PAGE, followed by heavy chain (HC) specific western blotting. This approach thereby allowed analysis of many constructs at the shake flask scale. Advantage was taken of the mutagenesis of C<sub>H</sub>1 to introduce a SpeI restriction site to facilitate rapid cloning  
35 of novel hinge sequences as annealed oligonucleotide cassettes. To introduce this site the Ser N-terminal to the interchain disulphide Cys in the heavy chain was changed to a Thr, hence changing the C-terminus of the C<sub>H</sub>1 from <sup>N</sup>KSCDKTHTCAA<sup>C</sup> to <sup>N</sup>KTSdkTHTCAA<sup>C</sup> (changes underlined).

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Bacterial strain and plasmid constructions - All final expression plasmids were based on pACYC184 [Yarranton, G.T. and Mountain A. (1992) in: Protein Engineering - a Practical Approach (Rees, AR., Stemberg, M.J.E. and Wetzel, R. eds) IRL Press, Oxford pp. 303-326) and used to transform W3110, wild type *E.coli* ATCC 27325.

A variant of A5B7g3 Fab' (graft number 3 of a humanised Fab' binding to CEA - carcinoembryonic antigen) lacking the interchain disulphide bond was first constructed. The interchain Cys codon in the light chain (LC) cKappa was changed by PCR mutagenesis to Ser using the mutagenic oligo:

5'-GCCGCGAATTCCGCACCTCTCCCTCTAACAGACTCTCCCCGTGAAGCTC-3'.

A similar strategy was used to remove the interchain Cys codon from the C<sub>H</sub>1 of the heavy chain (HC) and introduce the Spel restriction site using the mutagenic oligo:

5'-CCGCAAGCTTGGATCCTCATCACGCCGCATGTGTGAGTTTGTCACTAGTTT

15 TGGGCTCAACTTTC-3'

These and all subsequent clones were checked by DNA sequencing on an ABI 373A sequencer using PRISM cycle sequencing kit. Novel hinge sequences were produced by ligation of annealed oligonucleotide pairs with 5' Spel-HindIII 3' ends via a similarly restricted A5B7g3 HC only plasmid, followed by reconstitution of the final dicistronic expression plasmid.

The coding region for Fab' 40.4 (a humanised Fab' binding a human cytokine) cKappa was similarly altered using the mutagenic oligonucleotide

5'-GGCCTGAGCTCACCAAGTAACAAAAGCTTAATAGAGGAGAGTCTTGAGGAGGA

25 AAAAATGAAG-3'.

A restriction fragment for the original Fab' hinge (NCAAC) C<sub>H</sub>1 lacking the interchain disulphide bond cysteine was moved from A5B7g3 into the expression plasmid for Fab 40.4 as a restriction fragment. Since the Spel site in the Fab 40.4 final expression plasmid pDPH40 was unique it was possible to make further hinge variants rapidly by directly ligating annealed oligonucleotide pairs with 5' Spel-NotI 3' overhangs into similarly restricted pDPH40. Details of the hinge sequences and plasmids are shown in Table I. During DNA manipulation steps preferred codons for *E.coli* as defined by Wada, K.N. et al. *Nucleic Acids Res.* (1991) 19, 1981 were chosen.

35

Shake flask experiments - Shake flask experiments were conducted essentially as described previously except that these were single plasmid experiments and required Tetracycline at 10µgml<sup>-1</sup> [Humphreys, D.P. et al., *FEBS Lett.* (1996), 380, 194]. L-

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Broth was used for all experiments with A5B7, and in order to give the highest possible cell density, 2xTY was used for those with Fab 40.4. Redox active compounds were added as solids to a final concentration of 1mM when required. Samples were taken at 0, 1, 2, and 4 hours post-induction.

5

Fermentation - synthetic SM6 C media: (5.0 g/L  $(\text{NH}_4)_2\text{SO}_4$ , 3.312g/L  $\text{Na}_2\text{H}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 3.870g/L KCl, 1.0g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.0 g/L Citrate, 4.00g/l Citric acid, 0.05 g/L  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.02 g/L  $\text{ZnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.02 g/L  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.005 g/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.004 g/L  $\text{CoSO}_4 \cdot 6\text{H}_2\text{O}$ , 0.0967 g/L  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.0003 g/L  $\text{H}_3\text{BO}_3$ ,

10

0.0002 g/L  $\text{NaMoO}_4$ ). Glycerol was used as a carbon source at 3% (w/v) and MAZU was used as an antifoam at 0.02% (v/v). pH was controlled with 50% (v/v)  $\text{NH}_4\text{OH}$ . Fab' expression was induced by switching of the carbon source to lactose at 5% (w/w), cells were harvested 24-36 hours post-induction. Fab' yields were typically 100-150mg/L.

15

Western blotting - Samples equivalent to 2l of periplasmic fractions were diluted 5 fold with dH<sub>2</sub>O and boiled for 5 minutes in non-reducing SDS-PAGE loading buffer, before being electrophoresed on 4-20% Tris-glycine gels (Novex) at 125v for 1.75 hours. The proteins were transferred to a PVDF membrane (Novex) for 2 hours at

20

100mA. The membrane was blocked for 1 hour at room temperature with 50ml per membrane of 'blocking buffer' (PBS / 0.1% (v/v) Tween 20 / 2% (w/v) skimmed milk), before shaking at room temperature for 1 hour with 5ml per membrane of anti Fd antibody (Sheep IgG anti Human IgG(Fd), ref. PC075, The Binding Site, Birmingham, U.K.) at 1/1000 (v/v) in 'antibody buffer' (PBS / 0.1% (v/v) Tween 20 / 0.1% 'blocking buffer').

25

The membrane was washed extensively in PBS / 0.1% (v/v) Tween 20, before shaking at room temperature for 1 hour with 5ml per membrane of a donkey anti sheep HRP conjugated antibody (Rabbit F(ab')<sub>2</sub> anti Sheep IgG Fc fragment HRP conjugate, ref. 313-036-046, Jackson) at 1/1000 (v/v) in 'antibody buffer'. The membrane was washed extensively in PBS / 0.1% (v/v) Tween 20, before

30

development with DAB substrate (Pierce). LC cross reactive bands are shown by lanes 4 and 1 of Figures 1 and 2 respectively. Life size positive transparencies of blots were used for laser scanning densitometry on a Molecular Dynamics model 300A machine using ImageQuant software version 4.2. Di-Fab derived heavy chains (di-HC) separate from Fab derived HC (free HC) during electrophoresis. Quantitation of the relative intensities of the two easily identifiable bands in each lane gave an estimate of the % di-Fab formed. This assumes a relatively constant level of production of HC and its association with LC between different Fab' variants. The pTTO-1 derived plasmids used produce an excess of LC, and so free HC in the

35

periplasm is undetectable (Shauna West, personal communication). Di-HC of proteins lacking the interchain disulphide migrate with a mobility similar to a purified Fab' standard with the interchain disulphide (compare lane 11 and 3 of Figure 1). The total absorbance of each peak was quantified, and the % of di-Fab in each sample  
5 was calculated thus: absorbance of di-HC band + absorbance of di-HC band + absorbance of HC band.

**Fab' purification** - Periplasmic extracts were clarified by centrifugation at 25,000g for 30 minutes, the pH was increased to 6.5 with 2.5M Tris, and applied to a ProteinG 10 sepharose (GammaBind, Pharmacia Biotech) column pre-equilibrated with P.B.S. After washing with P.B.S. to remove unbound material, the Fab' related material was eluted by washing the column with 0.1M Glycine.Cl pH 2.7. The pH of the eluate was increased to neutral with 2.5M Tris for storage at 4°C, Fab' concentrations after purification were typically  $\leq 0.2 \text{ mg ml}^{-1}$ .

15 Plasmids were constructed with two copies of the repeat sequence TCPPCPA with between 0 and 5 spacing residues, see pDPH30-35, Table I (Fab' construct nomenclature shows the number of middle hinge sequence repeats, followed by Roman numerals to show the number of spacing residues, and whether the protein  
20 has or lacks the interchain disulphide bond forming cysteine. For example, pDPH34 expresses A5B7g3 hinge 2iv inter = graft 3 of A5B7 with two hinge repeats, spaced by four aminoacids and lacking the interchain disulphide bond. pDPH42 expresses Fab' 40.4 hinge 2o + Cys, has two hinge repeats, with no spacing residues and having the interchain disulphide bond). These were evaluated for their ability to form  
25 di-Fab's in shake flask cultures as judged by western blotting and compared against the hinge 1/2 and hinge 1 constructs. The results are shown in Table II.

Similar to that seen by others (ref. 7), the hinge 1/2 Fab' produced no detectable di-Fab' in shake flasks. 'hinge 1' and 'hinge 2o' produced a moderate amount of di-Fab'  
30 (7.3% and 4.8% respectively). These versions were then made in the more highly expressed Fab 40.4, to see if increasing the periplasmic protein concentration of these Fabs would effect both an increase in di-Fab' formation *in vivo*, and discriminate the relative merits of an increasing number of hinge repeats.

35 It was surprising to see that all of the A5B7 constructs with spacing residues between the hinge repeats produced no detectable di-Fab'. The increasing length and flexibility of these spacing regions could allow the second copy of the hinge repeat to fold back and form intra-HC disulphide bonds, thereby masking the cysteines from forming the

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desired inter-HC disulphide bonds. Although it is difficult to prove this assumption directly, two new constructs were designed using Fab 40.4 with reduced spacing/flexibility by removing one (Ala) or two (Ala-Thr) of the endogenous spacing residues between the rigid polyproline II helices. The results for these as shown in  
5 Table II were not favourable, so a Fab' 40.4 with three copies of the hinge, 'hinge 3o' was made

The results show that an increase in the number of copies of hinge repeats result in a steady decrease in the % of di-Fab' produced. This decrease in % di-Fab' can be  
10 presumed to be due to increasing toxicity of the protein in the periplasm as the proteins become more cysteine rich. The decrease in % di-Fab' correlated with decreased cell viability post-Fab' induction as shown by lower peak O.D.<sub>600</sub>'s and increased cell lysis at the later time points. The disulphide redox machinery in the *E.coli* periplasm is now well understood, but it is thought to be less well adapted to  
15 cope with proteins having complex disulphide arrangements than that of the endoplasmic reticulum (reviewed by Humphreys *et al.*, 1996 *ibid*). It seems likely that complex hinges are not well tolerated.

Reduction of the spacing between two hinge repeats does not increase di-Fab' formation. 'Fab' 40.4 inter hinge 2<sub>1</sub>' shows decreased di-Fab' compared to the 'hinge  
20 2o' variant (8.1 % ± 4.3 compared to 25.1 % 8.43 - see Table II). 'Fab' 40.4 inter hinge 2<sub>2</sub>' shows 49.8 % ±16.78 di-Fab'. This figure suggests a high level of di-Fab' production. In fact this protein appears unable even to produce substantial amounts of full length Fab', this is demonstrated by the increased number and intensity of  
25 proteolytic bands in lane no. 7 of the western blot shown in Figure 1. Hence there is a small amount of di-Fab' which is high relative to the amount of Fab', but very little of either protein is produced relative to 'hinge 1' and 'hinge 2o'. It can be postulated that removing one or both of these spacing residues makes the hinge regions so inflexible that the cysteines are forced into a conformation that makes them proteolytically exposed or reactive to native *E.coli* proteins. Since the unmodified 1 hinge gave the  
30 greatest di-Fab' yield *in vivo*, it was decided to test other hinge isotypes for their effectiveness.

Effect of hinge isotype - True hinge sequences for the IgG2, 3, and 4 could not be  
35 created, but rather the middle hinge of each was fused onto the existing IgG1 upper hinge. The hinges created are therefore called IgG2 'like'. Since long cysteine rich hinges are poorly tolerated, only the CPRC and the first of the three repeating sequences of the IgG3 middle hinge were used. The prime superscript is used to

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show that this IgG3 sequence was truncated. These constructs were analysed in the same way previously, and the results are shown in Table II.

The IgG3' like hinge was poorly tolerated and so gave an artifactualy high % di-Fab'  
5 measurement in the same way that hinge 2.2 did, an example of this proteolysis is  
shown in lane 4 of Figure 2. The long IgG3 hinge is thought to be highly flexible  
[Brekke, O.H. et al (1995) Immunol. Today 16, 85] and this is responsible for some of  
the properties of this isotype. In the periplasm this length of exposed peptide is likely  
to be susceptible to proteolysis, and this may be the reason for the poor level of  
10 production of this protein. The IgG 2 like hinge produced a reasonable amount of di-  
Fab' (20.8 % ± 3.58), but gave no increase over that seen before with the IgG 1  
derivatives. A relative inability of the IgG 4 like Fab' to produce di-Fab' (8.63 % ±  
1.41) was oberved. There is only one amino acid difference between the IgG 4 like  
15 hinge and the IgG1 hinge - CPSP relative to CPPC respectively. The results are  
consistent with previous work (ref. 22, 23), which found that IgG 4 was less able to  
form inter-HC disulphide bonds than IgG1. The presence of a serine between the two  
cysteines may allow increased flexibility of the middle hinge, and thereby formation of  
intra-HC disulphides which block inter-HC disulphide formation.

Effect of IgM and IgA tailpieces - IgM and IgA both have 18 amino-acid C-terminal  
20 extensions called secretory tailpieces that are involved in their polymerisation. The  
penultimate residue for both tailpieces is a cysteine. This and other HC cysteines,  
along with non-covalent interactions are known to be involved in polymer formation,  
although the exact disulphide organisation is not completely understood [Davis, A.C.  
25 et al., (1989), EMBO J. 8, 2519 and Wiersma, E.J. and Shulman, M.J. (1995) J.  
Immunol. 154, 5265]. The tailpiece has been added to all four IgG isotypes and  
effected their polymerisation even in the absence of the additional HC cysteines and  
non-covalently interacting residues found in IgM and IgA [Smith, R.I.F. et al (1995) J.  
30 Immunol. 154, 2226]. The wild type and tailpiece sequences described by  
Sørensen, V. et al., (1996) J. Immunol. 156, 2858] were added to Fab' 40.4  
containing a 'hinge 1' sequence. The 'hinge 1' sequence was chosen since it seemed  
possible that at least one free cysteine might be required to act as a mimic of Cys<sup>414</sup>  
of IgM to provide for tailpiece-HC, and one other to allow for direct inter-HC  
interactions such as thought to be provided for by Cys<sup>337</sup> of IgM. The sequences  
35 constructed in pDPH 50 and 51 are shown in Table I.

The Fab's were analysed by shake flask culture/western blotting for the presence of  
increased polymer formation. Although 'hinge 1' was known to give ~30% di-Fab'

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formation, it was found that the presence of either of the tailpieces completely abolished di-Fab' formation. No evidence was found for the formation of higher polymeric states as judged by western blots of non reducing native and SDS PAGE. It was assumed that the abolition of dimer formation inherent to 'hinge 1' was due to  
5 folding back of the tailpieces to form an intra-HC disulphide between the tailpiece Cys and one of those in the hinge. This seems quite possible as the tailpiece sequences are thought able to form one beta strand, allowing the tailpiece Cys to be close to the hinge [Pumphrey, R. (1986) Immunol. Today 7, 174].

10 In general, the results show that the presence of extra hinge cysteines above the single one found in the original Fab' was the most important factor in promoting di-Fab' formation *in vivo*, with 'Hinge 1' and 'hinge 2o' being the best sequences. No benefit was found with other versions of the 1 hinge over these, nor with any of the  
15 other 3 IgG isotypes tested. Simple hinges proved to be the most useful, with increasing length of hinge producing less % di-Fab' and a higher cell mortality. This was seen both with increasing copy number of the IgG1 hinge sequence i.e. hinge 1, 2o, and 3o, and with the long and flexible truncated IgG3.

20 More subtle structural differences also affected the efficiency of di-Fab' formation *in vivo*. Manipulation of the spacing residues between two copies of the hinge sequence '<sup>N</sup> TCPPCPA <sup>C</sup>' implied the importance of conformation for maintaining the hinge cysteines in a di-Fab' forming state. If the spacing region became too long, di-Fab'  
25 formation was completely abolished, presumably by looping back of the hinge masking hinge cysteines, whilst if the spacing was too short, the hinge was badly degraded *in vivo*.

30 Previous reports found variable production of di-Fab' from high cell density fermentaion of 5-70% using the hinge sequence <sup>N</sup>CPPCPCCPP<sup>C</sup> (ref. 9). The results herein demonstrate that it is very difficult to produce greater than 5-10 % di-Fab' *in vivo* from fermentations and that this is reproducible. In addition to the hinge sequence, these differences may be accounted for by differences in Fab' expression levels, fermentation conditions and host strain. The Fab' yield here was 5 to 10 fold lower than that described by Rodrigues *et al.*, (ref. 9). However, it is known that specific conditions can cause a spontaneous, high efficiency (~80%), and batch  
35 specific di-Fab' formation during purification. Such protein purification conditions were not used during these small scale experiments.

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In summary, di-Fab' formation *in vivo* in the periplasm of *E.coli* is an inefficient process that is modulated *inter alia* by hinge sequence and complexity. Two hinge sequences ('hinge 1' and 'hinge 2o') have been identified under *in vivo* selection conditions to be the most efficient for di-Fab' formation.

5

### EXAMPLE 2

#### Investigation of serum permanence times and hinge specific pegylation of F(ab')<sub>2</sub> molecules with modified hinges

##### Reagents

10 NEM,  $\beta$ -MA, DTDP, and Tris were from Sigma (UK) and of the highest grade available. Pyrogen free 'Flowfusor' water was used for chromatography (Fresenius, Basingstoke, UK). All other laboratory reagents were reagent grade from BDH (UK).

##### Production and Purification of Fab'

15 The Fab' used was 'Fab 40.4', as described in Example 1. Fab' fermentations and Protein G sepharose purifications (GammaBind Plus, Pharmacia Biotech) were as described in Example 1, with one modification. *E.coli* periplasmic proteins were extracted from fermentation cell paste by overnight incubation at 30°C and with shaking at 250 rpm in one fermentation harvest volume of 100mM Tris/10mM EDTA  
20 (pH 7.4). The conductivity and pH of the crude extract were altered to  $\leq$  3.5mS cm<sup>-1</sup> and  $\leq$  4.5 by addition of water and glacial acetic acid respectively. Crude extract was then passed over an 80ml (compacted) bed volume of Streamline SP cation exchange resin (Pharmacia Biotech) in expanded bed mode. The resin was pre-equilibrated with 50mM sodium acetate pH 4.5 in expanded bed mode. After  
25 extensive washing, bound material was eluted in compacted mode with 50mM sodium acetate/200mM NaCl pH 4.5. The pH of the peak fraction was increased to  $\geq$  6.5 with 2.5M Tris, and applied to a Protein G sepharose column pre-equilibrated with PBS. After washing with PBS to remove unbound material, the Fab' material was eluted with 0.1M Glycine Cl pH 2.7. The eluate was neutralised with 2.5M Tris  
30 for storage at 4°C.

##### Production and Purification of F(ab')<sub>2</sub>

Fab' material was concentrated and buffer exchanged to  $\geq$  12 mg ml<sup>-1</sup> in 0.1M sodium phosphate buffer pH 8.0 using ultra-filtration with a 10kDa cut-off membrane. Hinge thiols were activated and traces of F(ab')<sub>2</sub> depending upon final protein concentration) were removed by reduction with 9-mM  $\beta$ -MA in 0.1M sodium phosphate buffer pH 8.0 for 45 min. at 37°C. Reductant was removed by desalting on a G25M sephadex column (PD10), (Pharmacia Biotech) pre-equilibrated with

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0.1M sodium phosphate buffer pH 8.0. F(ab')<sub>2</sub> was allowed to form by incubation at room temperature overnight. Any remaining thiol groups were blocked by incubation with 10mM NEM in PBS before analysis by SDS-PAGE (4-20% Tris/glycine gels, Novex UK) and HPLC (GF-250 column equilibrated with 0.2M sodium phosphate buffer pH 7.0).

F(ab')<sub>2</sub> was separated from Fab' on a small scale by collecting preparative HPLC fractions (GF-250XL column equilibrated with 0.2M sodium phosphate buffer pH 7.0), or on a large scale using hydrophobic interaction chromatography (HIC). Solid 10 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to Fab'/F(ab')<sub>2</sub> mixtures to 0.75M before loading onto a phenyl-sepharose HP column (Pharmacia Biotech) equilibrated with 50mM phosphate buffer pH 7.0/0.75M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. After washing with equilibration buffer, bound material was eluted with 50mM sodium phosphate buffer pH 7.0. The dimeric species has a higher affinity for the HIC matrix than the monomeric Fab'. All purified 15 F(ab')<sub>2</sub> was judged to be 100% F(ab')<sub>2</sub> by HPLC and ≥ 95% F(ab')<sub>2</sub> by Coomassie stained SDS-PAGE.

Production of chemically cross linked F(ab')<sub>2</sub> (DFM)

DFM was produced as described previously using 1,6-bismaleimido-hexane (BMH) 20 cross-linker (ref. 2).

Resistance to reduction of F(ab')<sub>2</sub>

Purified F(ab')<sub>2</sub> at 0.25 mgml<sup>-1</sup> in 0.1M phosphate pH 7.0 was treated with β-MA from 0 to 2mM for 45min at 37°C. Reductions were stopped by addition of NEM to 10mM 25 and the relative amounts of F(ab')<sub>2</sub> and Fab' calculated by HPLC analysis.

Partial reduction of 'Hinge 2o' F(ab')<sub>2</sub> for hinge specific modification

Purified F(ab')<sub>2</sub> at 6mgml<sup>-1</sup> in 0.1M phosphate pH 6.0 was treated with β-MA from 0 to 2mM for 45 min. at 37°C. The reductant was removed by desalting with a P6 30 Biospin column (BioRad, UK) and samples taken immediately for thiol assay, NEM quenching and HPLC analysis and modification. 1.3mM β-MA was used for all preparative partial reductions.

Production and Cation Exchange Purification of F(ab')<sub>2</sub>

35 PEG-F(ab')<sub>2</sub> partially reduced as above was mixed with an equal volume of 25kDa linear PEG-maleimide in 0.1M phosphate pH6.0 (Shearwater Polymers Inc, Birmingham, Alabama, USA) to give a final molar excess of PEG:F(ab')<sub>2</sub> of 30 fold. After reacting overnight at room temperature, F(ab')<sub>2</sub>-PEG was separated from the

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- bulk of remaining F(ab')<sub>2</sub> using preparative scale HPLC. After concentration, and buffer exchange using a PD 10 column to 50mM sodium acetate pH 4.5, the F(ab')<sub>2</sub>-PEG/ unreacted PEG fraction was loaded on to a 1.6ml bed volume Poros HS column (PerSeptive Biosystems, Hertford, UK) on a BioCad Vision workstation. After 5 washing with 5 column volumes to remove unbound PEG-maleimide, F(ab')<sub>2</sub>-PEG was eluted with 50mM sodium acetate/ 40mM NaCl pH4.5, and F(ab')<sub>2</sub> was removed from the column with a linear gradient of 50mM sodium acetate pH 4.5 with NaCl from 40mM to 1M. Fractions were concentrated and buffer exchanged to 0.2M sodium-phosphate buffer pH 7.0 before estimating protein concentrations using A<sub>280</sub>.  
10 Protein was analysed by SDS-PAGE using 4-12% Tris-MES gels (Novex, UK).

#### Thiol assay

Thiols per F(ab')<sub>2</sub> were determined using DTDP as described previously (ref. 17).

- 15 Iodination of F(ab')<sub>2</sub>  
300µg of F(ab')<sub>2</sub> per animal group was <sup>125</sup>I-labelled using Bolton and Hunter reagent (Amersham) to a specific activity of 0.22-0.33 µCi/µg.

#### Mass Spectrometry

- 20 Molecular masses for Fab' was determined used Fisons VG Quattro triple quadrupole equipment in electrospray ionisation mode.

#### Animal studies

- 25 Male Sprague Dawley rats of 220-250g (Harlan) were injected *intra venously* with 20µg <sup>125</sup>I-labelled Fab 40,4 F(ab')<sub>2</sub> hinge variants whilst under Halothane anaesthesia (n=6 per group, except for 'hinge 20' where n = 10). Serial arterial bleeds from the tail were taken at 0.5, 2, 4, 6, 24, 48, 72 and 144 hours post administration. Samples were counted using a COBRA™ Autogamma counter (Canberra Packard). Data were plotted and Area Under Curve were calculated using 30 GraphPad Prism (GraphPad Software Incorporated) and is expressed as % injected dose hour (% id.hr). The t<sub>1/2</sub>β is defined by time points 24, 48, 72. The means and standard errors of means (SEM) of data are shown.

#### RESULTS

- 35 Production of F(ab')<sub>2</sub> *in vitro*

Three hinge sequences were used to produce F(ab')<sub>2</sub> *in vitro* : 'hinge 1/2'= NTHTCAAC; 'hinge 1' = NTHTCPPCPAC; and 'hinge 2o'= NTHTCPPCPATCPPCPAC. These contain 1, 2 and 4 hinge cysteines respectively.

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The 'hinge 1' and 'hinge 2o' proteins also contain one amino acid difference over 'hinge 1/2' of a Ser to Thr change in the C-terminus of CH1 as described in Example 1.

- After purification and concentration at pH 8.0 the Fab's were found to contain significant amounts of F(ab')<sub>2</sub>. In order for the F(ab')<sub>2</sub> product to be as homogeneous as possible the Fab' preparations were subjected to identical strongly reducing conditions (80mM β-MA, pH 8.0) so that the oxidation process started from 100 % Fab'. Antigen binding analysis (BIAcore) of F(ab')<sub>2</sub> show that these very strong reducing conditions do not affect Fab affinity for soluble antigen. Reduced and desalting Fab' was left at room temperature overnight for maximum F(ab')<sub>2</sub> yield.
- However, by taking samples during the oxidation for quenching with NEM it could be seen that approximately 60% of achievable F(ab')<sub>2</sub> forms within the first 60 minutes of the oxidation - see Figure 3. Both 'hinge 1/2' and 'hinge 2o' reached similar final yields of ~65% with an overnight incubation, however, F(ab')<sub>2</sub> formation within the first 30 minutes was more rapid for 'hinge 2o' than 'hinge 1/2'. This is probably a consequence of the greater number of hinge cysteines increasing the chances of Fab'-Fab' hinge cysteine interactions occurring. The yield of F(ab')<sub>2</sub> using 'hinge 2o' can be increased to 80% simply by increasing the concentration of Fab' to >20mgml<sup>-1</sup>.
- It was surprising that 'hinge 1' did not reach the same final yields of F(ab')<sub>2</sub> formation as the other two proteins. It is possible that after reduction a percentage of 'hinge 1' rapidly undergoes intra-hinge disulphide formation, thereby capping the thiols off from inter-hinge disulphide formation.
- No C-terminal proteolysis was observed with purified 'hinge 1' or hinge 2o' F(ab')<sub>2</sub>, removing the possibility that a proportion of 'hinge' molecules have a cysteine missing. HC from reduction of both 'hinge1' and 'hinge 2o' were shown by mass spectrometry to be full length molecules - 'hinge 1' had an observed mass of 24703.65 ± 0.55 Da relative to the predicted mass of 24705.12 Da, 'hinge 2o' had an observed mass of 25366.40 ± 6.13 Da relative to the predicted mass of 25374.88 Da.

Number of disulphide bonds in F(ab')<sub>2</sub> hinge regions

- 'Hinge 1/2' F(ab')<sub>2</sub> has an advantage over 'hinge 1' and 'hinge 2o' in that the product of hinge disulphide bond formation is homogeneous. In contrast 'hinge 1' may be able to form three different species of F(ab')<sub>2</sub> with a mixture of single and double disulphide and 'head to head' and 'head to tail' species (see Figure 4). The picture is potentially even more complex with 'hinge 2o'. Consideration of the primary sequence (in the absence of modelling predictions) shows a large number of species that could be

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formed. These differ in the number of disulphide bonds (between one and four), staggered linear arrangements, and gross quaternary structure (both 'head to head' and 'head to tail' formations). Rotation around a disulphide bond makes it seem unlikely that single disulphide bonded forms of 'hinge 2o' could survive more than  
5 transiently in the absence of thiol capping agents.

With F(ab')<sub>2</sub> produced *in vivo* in *E.coli*, the presence of more hinge cysteines does not result *per se* in a highly disulphide bonded hinge (ref. 9). Hence it was important to demonstrate whether the two larger hinges contained more than one disulphide bond.  
10 Advantage was taken of an observation made whilst optimising reduction/oxidation conditions that 'hinge 2o' required a higher β-MA concentration in order to reduce all of the contaminating F(ab')<sub>2</sub> in the purified Fab'. It was possible that the different purified F(ab')<sub>2</sub> proteins would exhibit differences in their ability to resist reduction to Fab'. It was found that at 0.25 mg ml<sup>-1</sup> and pH 7.0 'hinge 1/2' is completely reduced by  
15 125μM β-MA (see Figure 5). At the same concentration of β-MA 68.3% of the 'hinge 1' preparation is still dimeric. This implies that approximately 68% of the population of 'hinge 1' F(ab')<sub>2</sub> has two disulphide bonds. At the slightly greater β-MA concentration of 166μM, 100% of the 'hinge 2o' preparation is still dimeric. Hence by extrapolation, >68% of 'hinge 2o' molecules must have more than 2 disulphide bonds i.e. 3 or 4.  
20 Although hinge structural differences might have an effect on the accessibility or chemical reactivity of hinge disulphides, it seems unlikely that they would be significant enough to cause the gross differences seen in Figure 5. Hence 'hinge 1' and 'hinge 2o' have a high degree of multiple disulphide bonding.

25 Effect of hinge sequence of F(ab')<sub>2</sub> pharmacokinetics in Rat

F(ab')<sub>2</sub> made from all three hinge constructs along with a DFM control were <sup>125</sup>I-labelled so that their pharmacokinetics could be followed in a rat model. The data shown in Table III support the comparisons made *in vitro* between hinges 1/2, 1, and 2o. F(ab')<sub>2</sub> with a single hinge disulphide 'hinge 1/2' is cleared most rapidly from the circulation, with an AUC of 166.2 ± 9.8% injected dose. hour (% id.hr). F(ab')<sub>2</sub> with an identical protein sequence, but linked by thioether bonds is cleared more slowly, AUC = 384.2 ± 32.1 (% id.hr). This is interpreted as evidence of the greater lability of the disulphide over thioether linkage, leading to a more rapid breakdown *in vivo* to Fab'. The smaller Fab' is excreted more rapidly through the kidneys than F(ab')<sub>2</sub>. Increase  
30 of the average number of disulphide bonds in the hinge with 'hinge 1' and 'hinge 2o'  
35 results in increased AUC (423.6 ± 35.0 and 509.7 ± 31.3 (%id.hr) respectively) over that of 'hinge 1/2'. The curves for clearance from circulation of rats for the four F(ab')<sub>2</sub> molecules tested are shown in Figure 6. This demonstrates graphically that the major

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effect of the different hinge stabilities appears to be on the  $\alpha$ -phase. The initial clearance of 'hinge 1/2'  $F(ab')_2$  is much more rapid than the other three  $F(ab')_2$  molecules which are more grouped together. Presumably increased resistance of 'hinge 1', 'hinge 2o' and DFM  $F(ab')_2$  to reductive or proteolytic forces in circulating blood leads to the molecules surviving for longer as the larger  $F(ab')_2$  species which is cleared more slowly from the circulation.

Partial Reduction and PEGylation of 'Hinge 2o'  $F(ab')_2$  in vitro

Since the 'hinge 2o'  $F(ab')_2$  has a large proportion of molecules with multiple hinge disulphides, it seemed possible that a number of disulphide bonds could be broken and uncoupled thiols activated whilst retaining the molecule as a dimeric species. Such thiols would be particularly useful for attachment of effector or reporter groups such as radionucleotides, toxins, or PEG. Covalent attachment of PEG to proteins is attractive since it is a simple route to increasing the serum permanence, reducing immunogenicity, and decreasing proteolysis *in vivo*.

Using a range of  $\beta$ -ME concentrations at pH 6.0 and analysis of NEM quenched samples of HMPc it was found that at  $\leq 1.8$ mM  $\beta$ -ME 100% of the  $F(ab')_2$  population remained dimeric. This figure may vary between batches of  $F(ab')_2$  due to hinge 20 disulphide occupancy heterogeneity. At 1.3mM  $\beta$ -ME thiol assay with DTDP showed  $1.034 \pm 0.090$  thiols per  $F(ab')_2$  being liberated for reaction.  $F(ab')_2$  not treated with  $\beta$ -ME showed little free thiols per  $F(ab')_2$ . The efficiency of PEGylation of  $F(ab')_2$  was calculated after purification of  $F(ab')_2$ -PEG by cation exchange and estimation of protein concentration by  $A_{280}$  to be  $\leq 1.3\%$ .

**Table I.** Oligonucleotide cassettes for construction of hinge sequences.

## **Plasmid name and oligonucleotide cassette and hinge sequence**

**pDPH228 ASB7g3 hinge ½ Δinter**  
**5'- ACT AGT GAC AAA ACT CAC ACA TGC GCC GCG TGA TGA GGA TCC AAG CTT**  
**T S D K T H T C A A \***

pDPH29 ÁSB7g3 hinge I Ainter  
 5: CT AGT GAC AAA ACT CAC ACC TGC CCG CCG TGA GGA TCC A  
 3: A CTG TTT TGA GTG AGC GGC ACG GGC CCT ACT AGG TTC GA S:  
 T S D K T H T C P P C P A \*

**pDPH30-ASB7g3 hinge 20 Δinter**

5'-	CT	GAC	AAA	ACT	CAC	ACC	TGC	CCC	TGG	CGC	TGC	CGG	TGA	TGA	GGA	TCC	A	3'				
3'-	CA	CTG	TTT	TGA	TGT	ACG	GCG	ACG	GCG	CGC	TGG	ACG	GCG	ACG	GCG	CGC	ACT	CCT	AGG	TTC	GA	5'
	T	S	D	K	T	H	T	C	P	P	C	P	A	T	C	P	C	P	A	*	*	

**pDPH31 ASB783 hinge 2i Δinter**  
 5' CT AGT GAC AAA ACT CAC ACC TGC CGG CCC TGC CCG GGA ACC TGC CCG TGC CCG GCG TGA TCC A  
 3' CA CTG TTT TGA GTG TGG ACG GGC GGC CCT TGA ACG GGC GGC ACT CCT AGG TTC GA 5'

**pDPH32 ASB7g3 hinge 21 Dipter**  
 5' CT AGT GAC AAA ACT CAC ACC TGC CCG CCG TGC CGG GGA AC C TGC CGG GCG TGC CGG GCA TCC TGA CGA 3'  
 3' CA CTG TTT TGA GTG TGG ACG GGC ACG GGC CCT GGA ACG GGC GGC ACT CCT AGG TTC GA 5'

Table 1 (continued)

**pDPH34 ASB7g3 hinge 2iv Δinter**  
 5'- CT AGT GAC AAA ACT CAC ACC TGC CGG CCG TGC CCG GGA GGA ACC TGC CCG TGC TGA TGA GGA  
 3'- CA CTG TTT TGA GTG ACG GGC GGC ACG CCC CCT GGA GGA GCA TGG ACG GGC ACG GGC CGC ACT ACT CCT  
 T S D K T H T C P P C P A G G T C P C C P A \*  
 TCC A 3'  
 AGG TTC GA 5'

**pDPH35 A5B7g3 hinge 2' Δinter**  
 5'- CT AGT GAC AAA ACT CAC ACC TGC CCC CGG AAA GGC GAA ACC TGC CCG TGC TGC CGG TGA TGA  
 3'- CA CTG TTT TGA GTG TGG AGC GGC GGC ACG CGC CGC TTT CCG CTT TGG ACG GGC ACG GGC CGC ACT ACT  
 T S D K T H T C P P C P A K G K E T C P C P A \* \*

**DPH40 Fab 40.4 hinge Y<sub>2</sub> Δinter**

5:	CT	GAC	AAA	ACT	CAC	ACA	TGC	ACC	GCG	TGA	TGA	GGA	TCC	AAG	CTT	GC	3'
3:	A	CGC	TTC	TGA	GTC	TGT	ACG	CGG	CGC	ACT	ACT	CCT	AGG	TTC	GAA	CAC	5'
	T	S	D	K	T	H	T	C	A	*	*	*	*	*	*	*	

**pDPH41 Fab 40.4 hinge 20 Dimer**

5'	CTG	GAC	AAA	ACT	CAC	ACA	TGC	CCG	TGC	CCG	ACC	TGC	CCG	TGC	CCG	TGA	TGA	GGA	TCC	A	3'	
4'	A	CTG	TTT	TGA	GTC	TGT	ACG	GCG	GCG	GCG	ACG	GCG	GCG	ACG	GCG	ACT	ACT	CCT	AGG	TTC	GA	5'
3'	T	S	D	K	T	H	T	C	P	P	A	T	C	P	P	C	P	A	*	*		

Table 1 (continued)

**pDPH42 Fab 40.4 hinge 20 +Cys**  
<sup>5</sup> ACT TGT GAC AAA ACT CAC ACA TGC CCG CCG TGC CCG ACC TGC CCC TCC CGG CCG TGA TGA GGA TCC A 3'

pDPH44 Fab 40.4 hinge I Ainter  
 5. CT AGT GAC AAA ACT CAC ACA TGC CCA CCG TGC TGA CGA TCC A  
 3. A CTG TTT TCA GTG TGT ACG GCG GGC ACT ACT CCT AGG TTC GA S.

**pDPH52** F1b 40.4 hinge I +Cys  
**5.** ACT TGT GAC AAA ACT CAC ACA TCC CCG TGC CCG TGA TGA CGA TCC A  
 T C D K T H T C P P C P A \*

pDPH53 Fab 40.4 hinge 2,1 Dinter  
 5' CT AGT GAC AAA ACT CAC ACA TGC CCG CCG TGC CCC ACC TGC CCG TGC CCG TGA TGA GGA TCC A  
 3' A CTG TTT TGA GTG TGT ACG GGC GGC ACG GGC GGC TGG ACG GGC GGC ACG GGC CGC ACT CCT AGG TTC GA 5'

Table 1 (continued)

pDPH54 Fab 40.4 hinge 2<sub>2</sub> Dimer  
 5' CT AGT GAC AAA ACT CAC ACA TGC CCC CCG TGC CCA CGG TCA TGA GGA TCC A  
 3' A CTG TTT TGA GTG TGT AGC GGC GGC ACG GGC CGC ACT ACT CCT AGG TTC GA 5'

**pDPH50 Fab 40.4 hinge 1 Dimer IgMip**  
 S: CT AGT GAC AAA ACT CAC ACC TGC CCG CCG TGC CGG CCG AAC CTT GAG AGC GAT  
 S: CA CTG TTT TGA GTC TGG AGC GGC AGC TCG GAC ATA TTG CAC TAC TCG CTA  
 T P S D K T H T C P P C P A G K P T L Y N V S L V M S D  
 ACC GCG GCG ACC TGT TAT TGA TGA GGA TCC AAG CTT GC 3'  
 TGG TCT A G C T C Y \* \* \*  
 5' CGG CCG TGG ACA ATA ACT CCT AGG TTC GAA CGG CGG 5'

**pDPH51 Fab 40.4 hinge I Dinter IgA1p**  
**5'-CT AGT GAC AAA ACT CAC ACC TGC CCC TGC CCG CCG GCG GCG AAA AAC GTC AGC GTG GTG ATG**  
**3'-CA CTG TTT TGA GTC TGG ACG GGC GGC CGC CGC CGC TTT GGC TCG GTA CAC TCG CAC TAC CGC TAC**  
**T S D K T H T C P P C P A G K P T H V N V S V V . M**

Table 1 (continued)

pDPH61 Fab 40.4 'IgG2 like' hinge Δinter  
 5': CT AGT GAC AAA ACT CAC ACC TGC TGC TGC GAA TGC CCG CCG TGC CGT GCG TGA GGA TCC AAG CTT GC 3'  
 3': A CTG TTT TGA GTC TGG AGC ACG CAC CCT ACG GGC GGC AGC GGC CGC ACT ACT CCT AGG TTC GAA CCC CGG 5'  
 \* T S D K T H T C V C P C P C P A \*

pDPH62 Fab 40.4 'IgG3' like' hinge Δinter  
 5': CT ACT GAC AAA ACT CAC ACC TGC TGC CGT TGC CGG GAA CGG AAA AGC TGC GAT ACC CCG CCG CCG TGC CGT TGC CCG GCG  
 3': A CTG TTT TGA GTC TGG AGC ACG GGC GCA AGC GGC CCT GGC TTT TCG AGC CTA TGG GGC AGC GCA AGC GGC CGC  
 \* T S D K T H T C V C P R C P K S C D T P R C P C P A \*

TGA TGA GGA TCC AAG CTT GC  
 ACT ACT CCT AGG TTC GAA CGC CGG 5'

pDPH63 Fab 40.4 'IgG4 like' hinge Δinter  
 5': CT AGT GAC AAA ACT CAC ACC TGC TGC CGG AGC TGC CGG TGA TGA GGA TCC AAG CTT GC 3'  
 3': A CTG TTT TGA GTC TGG AGC GGC TCG AGC GGC CCT AGG ACT CCT AGG TTC GAA CGC CGG 5'  
 \* T S D K T H T C V C P S C P A \*

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**Table II**

**Effect of hinge sequences on efficiency of di-Fab formation *in vivo* in shake flask experiments.**

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		% di-HC
A5B7 Δinter, hinge ½	TSDKTHTCAA	Non det.
A5B7 Δinter, hinge 1	TSDKTHTCPPCPA	$7.375 \pm 2.338$
A5B7 Δinter, hinge 2o	TSDKTHTCPPCPATCPPCPA	$4.804 \pm 3.23$
A5B7 Δinter, hinge 2i	TSDKTHTCPPCPAGTCPPCPA	Non det.
A5B7 Δinter, hinge 2ii	TSDKTHTCPPCPAGGTCPPCPA	Non det.
A5B7 Δinter, hinge 2iii	TSDKTHTCPPCPAGGGTCPPCPA	Non det.
A5B7 Δinter, hinge 2iv	TSDKTHTCPPCPAGGGGTCPCCPA	Non det.
A5B7 Δinter, hinge 2v	TSDKTHTCPPCPAKGKGETCPPCPA	Non det.
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Fab 40.4 Δinter, hinge ½	TSDKTHTCAA	Non det.
Fab 40.4 Δinter, hinge 1	TSDKTHTCPPCPA	$35.7 \pm 4.85$
Fab 40.4 Δinter, hinge 2o	TSDKTHTCPPCPATCPPCPA	$25.1 \pm 8.43$
Fab 40.4 Δinter, hinge 3o	TSDKTHTCPPCPATCPPCPATCPPCPA	$20.8 \pm 3.58$
Fab 40.4 Δinter, hinge 2.1	TSDKTHTCPPCPCTCPPCPA	$8.1+ \pm 4.3$
Fab 40.4 Δinter, hinge 2.2	TSDKTHTCPPCPCTCPPCPA	$49.8 \pm 16.78$
Fab 40.4 hinge 1 + Cys	TCDKTHTCPPCPA	$14.4 \pm 9.75$
Fab 40.4 hinge 2o + Cys	TCDKTHTCPPCPATCPPCPA	$16.3 \pm 2.30$
Fab 40.4 'IgG2 like' hinge Δinter	TSDKTHTCCVECPPCPA	$20.8 \pm 5.95$
Fab 40.4 'IgG3' like' hinge Δinter	TSDKTHTCPRCPEPKSCDTPPPCPRCPA	$29.3 \pm 5.95$
Fab 40.4 'IgG4 like' hinge Δinter	TSDKTHTCPSCPA	$8.63 \pm 1.41$

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**Table III****Effect of hinge composition on F(ab')<sub>2</sub> pharmacokinetics in rat.**

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No. hinge disulphides	AUC (% Id.Ihr)	SEM (% Id.Ihr)	t <sub>1/2α</sub> (hr)	SEM (hr)	t <sub>1/2β</sub> (hr)	SEM (hr)	n
<b>Fab 40.4 F(ab')<sub>2</sub> 'hinge ½'</b>	1 166.2	9.8	1.3	0.04	19.0	1.8	6
<b>Fab 40.4 F(ab')<sub>2</sub> 'hinge 1'</b>	≤ 2 423.6	35.0	2.1	0.1	13.1	0.2	6
<b>Fab 40.4 F(ab')<sub>2</sub> 'hinge 2o'</b>	≤ 4 509.7	31.4	3.0	0.2	11.0	0.5	10
<b>Fab 40.4 DFM</b>	1 thioether 384.2	32.1	2.3	0.1	16.4	0.3	6

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## FIGURE LEGENDS

### **Figure 1. Effect of hinge sequence and complexity of di-Fab' formation *in vivo*.**

Anti-Fd western blot of 4-20% Tris-glycine SDS-PAGE, reducing for lanes 1 and 2,

- 5 non-reducing for lanes 3 to 12. Molecular size of standards (kDa) are shown on the right. All periplasmic samples loaded equally, except LC only control (lane 4) which has a 5 fold excess. Lane 1, pDPH52 Fab 40.4 hinge 1 + cys; lane 2, pDPH44 Fab 40.4 hinge 1 inter; lane 3, purified Fab' 40.4 + cys control; lane 4, Fab 40.4 light chain only control; lane 5, pDPH42 Fab 40.4 hinge 2o + cys; lane 6, pDPH52 Fab 40.4 hinge 1 + cys; lane 7, pDPH54 Fab 40.4 hinge 2<sub>o</sub> inter; lane 8, pDPH53 Fab 40.4 hinge 2<sub>o</sub> inter; lane 10, pDPH41 hinge 2o inter; lane 11, pDPH44 hinge 1 inter; lane 12, pDPH40 hinge inter.
- 10
- 11
- 12

### **Figure 2. Comparison of effect of hinge isotype on di-Fab' formation *in vivo*.**

15 Anti-Fd western blot of 4-20% Tris-glycine non-reducing SDS-PAGE. Molecular size of standards (kDa) are shown on the right. All periplasmic samples loaded equally, except

- standards (kDa) are shown on the right. All periplasmic samples loaded equally, except LC only control (lane 1) which has a 5 fold excess. Lane 1, Fab 40.4 light chain only control; lane 2, purified Fab' 40.4 + cys control; lane 3, pDPH63 Fab 40.4 'IgG4 like' hinge inter; lane 4, pDPH62 Fab 40.4 'IgG3 like' hinge inter; lane 5, pDPH62 Fab 40.4 'IgG2 like' hinge inter; lane 6, pDPH44 Fab 40.4 hinge 1 inter (IgG1).
- 20

### **Figure 3. Effect of hinge sequences and duration of oxidation on F(ab')<sub>2</sub> formation *in vitro*.**

The mean and SD of three independent experiments conduction at pH 8.0 and room temperature are shown.

25

### **Figure 4. Range of F(ab')<sub>2</sub> molecules capable of being formed**

- A. F(ab')<sub>2</sub> formed by 'hinge 1', and B, F(ab')<sub>2</sub> formed by 'hinge 2o', only the 'head to head' forms are shown for 'hinge 2o', hence the use of 'x2' to denote possible 'head to tail' forms.

30

### **Figure 5. Effect of hinge sequence on resistance of F(ab')<sub>2</sub> to reduction *in vitro***

F(ab')<sub>2</sub> at 0.25mgml<sup>-1</sup> are subjected to reduction at pH 7.0, 37°C for 45 min. at the range of b-MA concentrations shown.

35

### **Figure 6. Effect of hinge composition on F(ab')<sub>2</sub> pharmacokinetics in rat.**

The mean and standard error of mean for each F(ab')<sub>2</sub> are shown. There were six animals in each group, except 'hinge 2o' where n=10.

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REFERENCES

1. Covell, D. G., J. Barbet., O. D. Holton., C D. V. Black., R. J. Parker, and J. N. Weinstein. 1986. Pharmacokinetics of monoclonal Immunoglobulins G1, F(ab')<sub>2</sub>, and Fab' in Mice. *Cancer Research* 46:3969.
- 5
2. King, D. J., A. Turner., A. P. H. Farnsworth., J. R. Adair., R. J. Owens., B. Pedley., D. Baldock., K. A. Proudfoot., A. D. G. Lawson., N. R. A. Beeley., K. Millar., T.A. Millican., B. A. Boyce., P. Antoniw., A. Mountain., R. H.J3. Begent., D. Shochat, and 10 G. T. Yarranton. 1994. Improved tumour targeting with chemically cross-linked recombinant antibody fragments. *Cancer Research* 54:6176.
- 15
3. Kaku, S., S. Yano., T. Kawasaki., Y. Sakai., K. I. Suzuki., K. Kawamura., Y. Masuho., N. Satoh., T. Takenaka., N. F. Landolfi, and M. S. Co. 1996. Comparison of the antiplatelet agent potential of the whole molecule, F(ab')<sub>2</sub> and Fab fragments of humanized anti-GPIIb/IIIa monoclonal antibody in Monkeys. *General Pharmacology* 27:435.
- 20
4. Plückthun, A. 1992. Mono- and bivalent antibody fragments produced in *Escherichia coli*: engineering, folding and antigen binding. *Immunological Reviews* 130:151.
- 25
5. George, A. J. T, and A. Epenetos. 1996. Advances in antibody engineering. *Expert Opinion in Therapeutic Patents* 6:441.
6. Melton, R. G. 1996. Preparation and purification of antibody-enzyme conjugates for therapeutic applications. *Advanced Drug Delivery Reviews* 22:289.
- 30
7. Carter, P., R. F. Kelley., M. L. Rodrigues., B. Snedecor., M. Covarrubias., M.D. Velligan., W. L. T. Wong., A. M. Rowland., C. E. Kotts., M. E. Carver., M. Yang, and A. et al. 1992. High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Bio/Technology* 10:163.
- 35
8. Better, M., S. L. Bernhard., S. P. Lei., D. M. Fishwild., 3. A. Lane., S. F. Carroll, and A. H. Horwitz. 1993. Potent anti-CD5 ricin A chain immunoconjugates from bacterially produced Fab' and F(ab')<sub>2</sub>. *Proceedings of the National Academy of Sciences, U.S.A.* 90:457.

-34-

9. Rodrigues, M. L., B. Snedecor., C. Chen., W. L. T. Wong., S. Garg., G. S. Blank., D. Maneval, and P. Carter. 1993. Engineering Fab' fragments for efficient F(ab')<sub>2</sub> formation in *Escherichia coli* and for improved *in vivo* stability. *Journal of Immunology* 152:6954.
- 5  
10. Pack, P., and A. Plückthun. 1992. Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in *Escherichia coli*. *Biochemistry* 31:1579.
- 10 11. Ducancel, F., D. Gillet., A. Carrier., E. Lajeunesse., A. Menez, and J. C. Boulain. 1993. Recombinant colorimetric antibodies: construction and characterization of a bifunctional F(ab')<sub>2</sub>/alkaline phosphate conjugate produced in *Escherichia coli*. *Bio/Technology* 11:601.
- 15 12. Speck, R. R., J. R. Couto., S. G. Godwin., R. B. Christian., R. Kiwan., R. L. Ceriani, and J. A. Peterson. 1996. Inverted Fab2s (IFab2s): engineering and expression of novel, dimeric molecules, with a molecular weight of 100 000. *Molecular Immunology* 33:1095.
- 20 13. Rheinheimer, M., C. Hardt., L. L. hag., P. Kufer., R. Gruber., A. Hoess., A. Lupas., C. Rottenberger., A. Pluckthun, and P. Pack. 1996. Multivalent antibody fragments with high functional affinity for a tumour-associated carbohydrate antigen. *Journal of Immunology* 157:2989.
- 25 14. Glennie, M. J.; H. M. McBride., A. T. Worth, and G. T. Stevenson. 1987. Preparation and performance of bispecific F(ab')<sub>2</sub> antibody containing thioetherlinked Faby fragments. *Journal of Immunology* 139:2367.
- 30 15. King, D. J., J. R. Adair., S. Angal., D. C. Low., K. A. Proudfoot., J. C. Lloyd., M. W. Bodmer, and G. T. Yarranton. 1992. Expression, purification and characterization of a mouse-human chimeric antibody and chimeric Fab' fragment. *Biochemical Journal* 281:317.
- 35 16. Worrell, N. R., A. J. Cucumber., G. D. Parnell., A. Mirza., J. A. Forrester, and W. C. J. Ross. 1986. Effect of linkage variation on pharmacokinetics of ricin A chain-antibody conjugates in normal rats. *Anti-Cancer Drug Design* 1:179.

-35-

17. Lyons, A., D. J. King., R. J. Owens., G. T. Yarranton., A. Millican., N. R. Whittle, and J. R. Adair. 1990. Site-specific attachment to recombinant antibodies via introduced surface cysteine residues. *Protein Engineering* 3:703.
- 5 18. Moroder, L., G. Hübener., S. Göhring-Romani., W. Göhring., H. J. Musiol, and E. Wünsch. 1990. Fully synthetic immunogens. Part I. Kinetic studies on air oxidation of the human IgG1 bis-cysteinyl fragment 225-232. *Tetrahedron* 46:3305.
- 10 19. Harris, L. J., S. B. Larson., K. W. Hasel., 3. Day., A. Greenwood, and A. McPherson. 1992. The three-dimensional structure of an intact monoclonal antibody for canine lymphoma. *Nature* 360:369.
- 15 20. Burton, D. R, and J. M. Woof. 1992. Human antibody effector function. *Advances in Immunology* 51:1.
21. Buchegger, F., A. Pelegrin., N. Hardman., C. Heusser., 3. Lukas., W. Dolci, and J. P. Mach. 1992. Different behaviour of mouse-human chimeric antibody F(ab')<sub>2</sub> fragments of IgG<sub>1</sub>, IgG<sub>2</sub>, and IgG<sub>4</sub> sub-class in vivo. *International Journal of Cancer* 50:416.
- 20 22. Angal, S., D. J. King., M. W. Bodmer., A. Turner., A. D. G. Lawson., G. Roberts., B. Pedley, and J. R. Adair. 1993. A single amino acid substitution abolishes the heterogeneity of chimeric mouse/human (IgG4) antibody. *Molecular Immunology* 30:105.
- 25 23. Bloom, J. W., M. S. Madanat., D. Marriott., T. Wong, and S. Y. Chan. 1997. Intrachain disulfide bond in the core hinge region of human IgG4. *Protein Science* 6:407.
- 30 24. Yoshimori, T., T. Semba., H. Takemoto., S. Akagi., A. Yamamoto, and Y. Tashiro. 1990. Protein disulfide-isomerase in rat exocrine pancreatic cells is exported from the endoplasmic reticulum despite possessing the retention signal. *Journal of Biological Chemistry* 265:15984.

**CLAIMS****1. A peptide of Formula (1)**

5                   **NTCPPCPXYCPPCPAC (1)**

wherein X and Y, which may be the same or different, is each a neutral aliphatic L-amino acid residue, and protected and reactive derivatives thereof.

10    **2. A peptide according to Claim 1 wherein X is an alanine residue.**

**3. A peptide according to Claim 1 or Claim 2 wherein Y is a threonine residue.**

15    **4. A protein comprising one polypeptide chain characterised in that said chain contains an amino acid sequence NTCPPCPXYCPPCPAC wherein X and Y are as defined in Claim 1.**

20    **5. A protein comprising two polypeptide chains characterised in that each of said chains contains an amino acid sequence NTCPPCPXYCPPCPAC wherein X and Y are as defined in Claim 1 and each chain is covalently linked to the other through one, two, three or four of the cysteine residues present in each of said amino acid sequences.**

25    **6. A protein according to Claim 4 or Claim 5 which is an antibody or an antigen binding fragment thereof.**

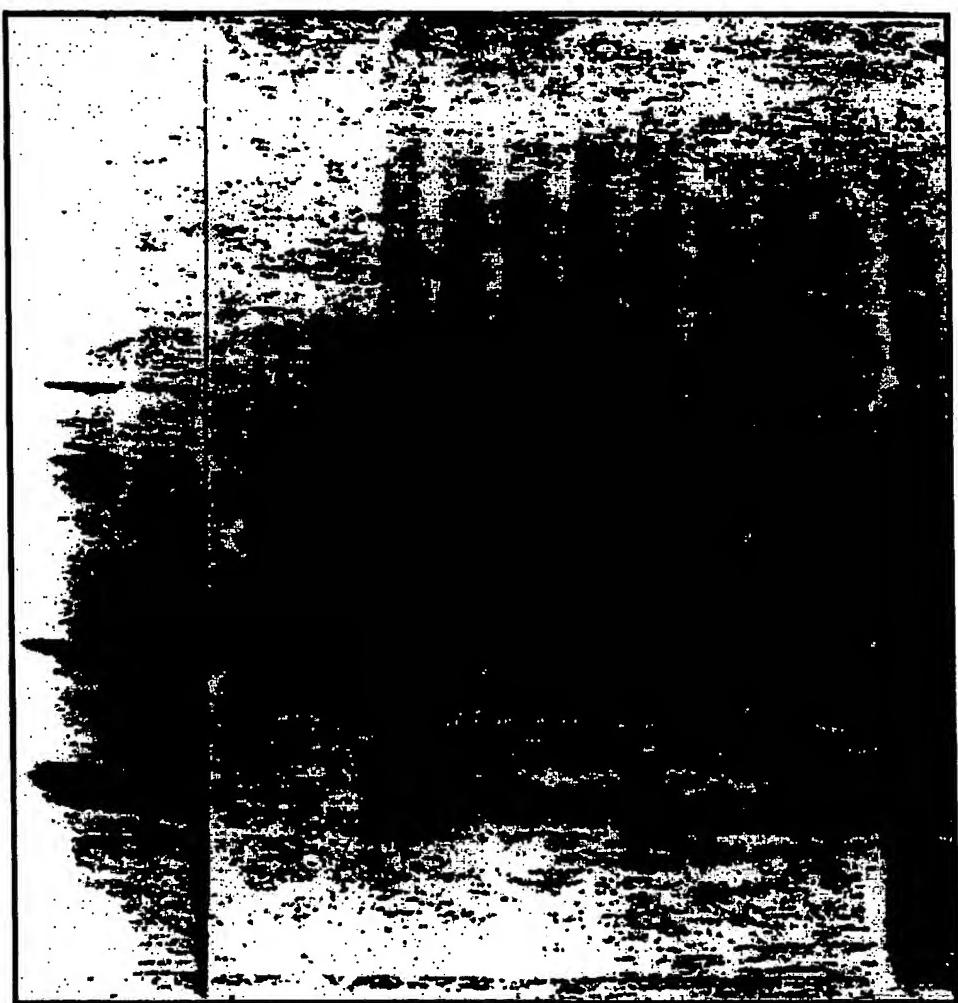
**7. A protein according to Claim 6 which is a Fab or Fab' fragment.**

30    **8. A protein according to any one of Claim 4 to Claim 7 which has one or more effector or reporter molecules attached to it.**

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**FIG. 1**

1 2 3 4 5 6 7 8 9 10 11 12



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FIG. 2

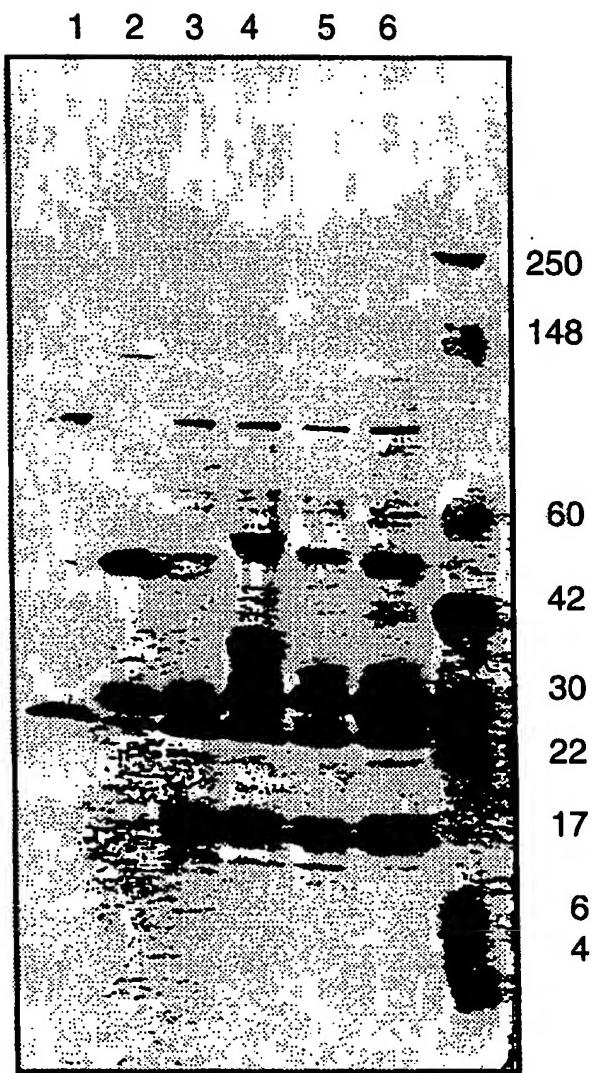
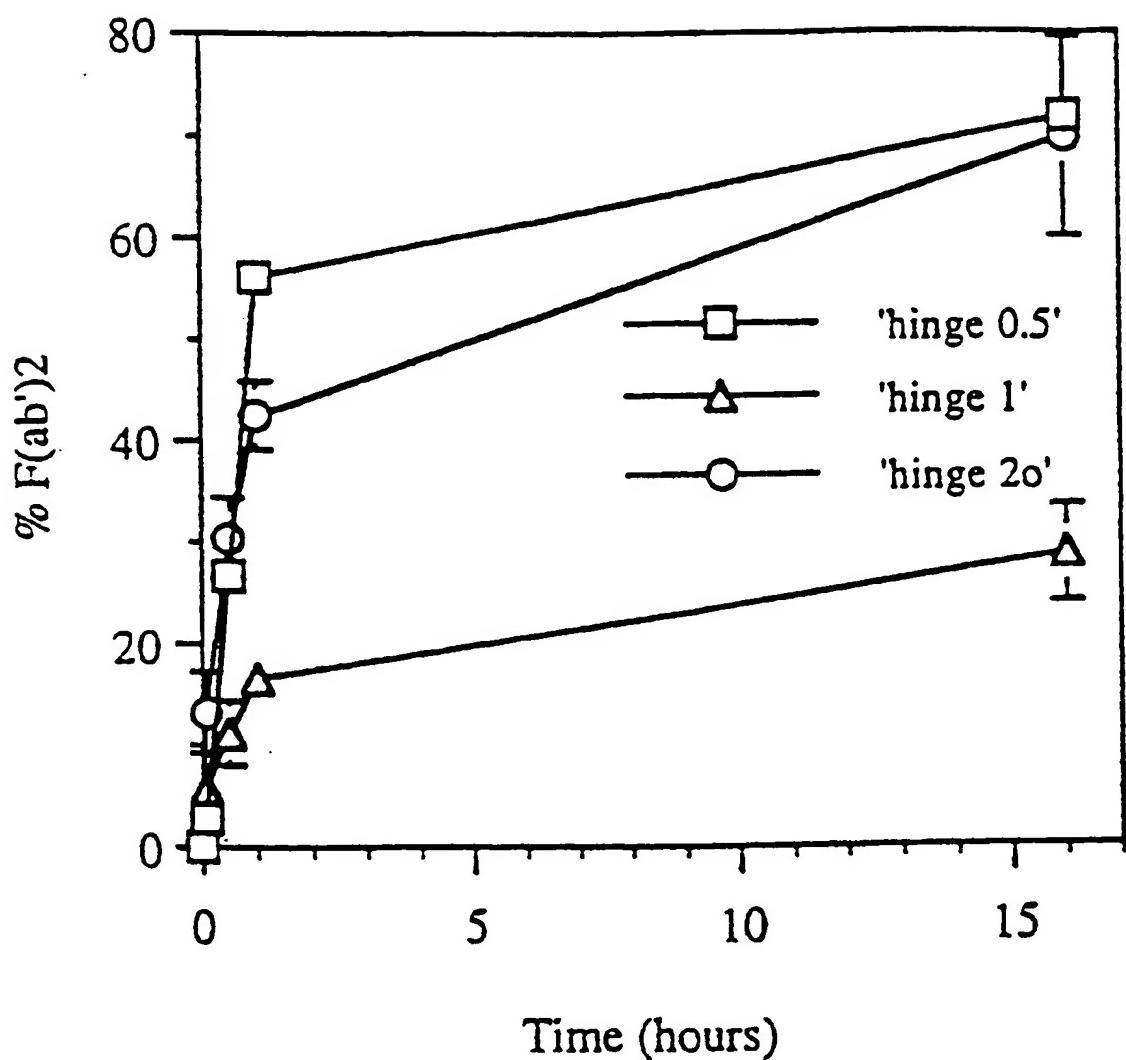
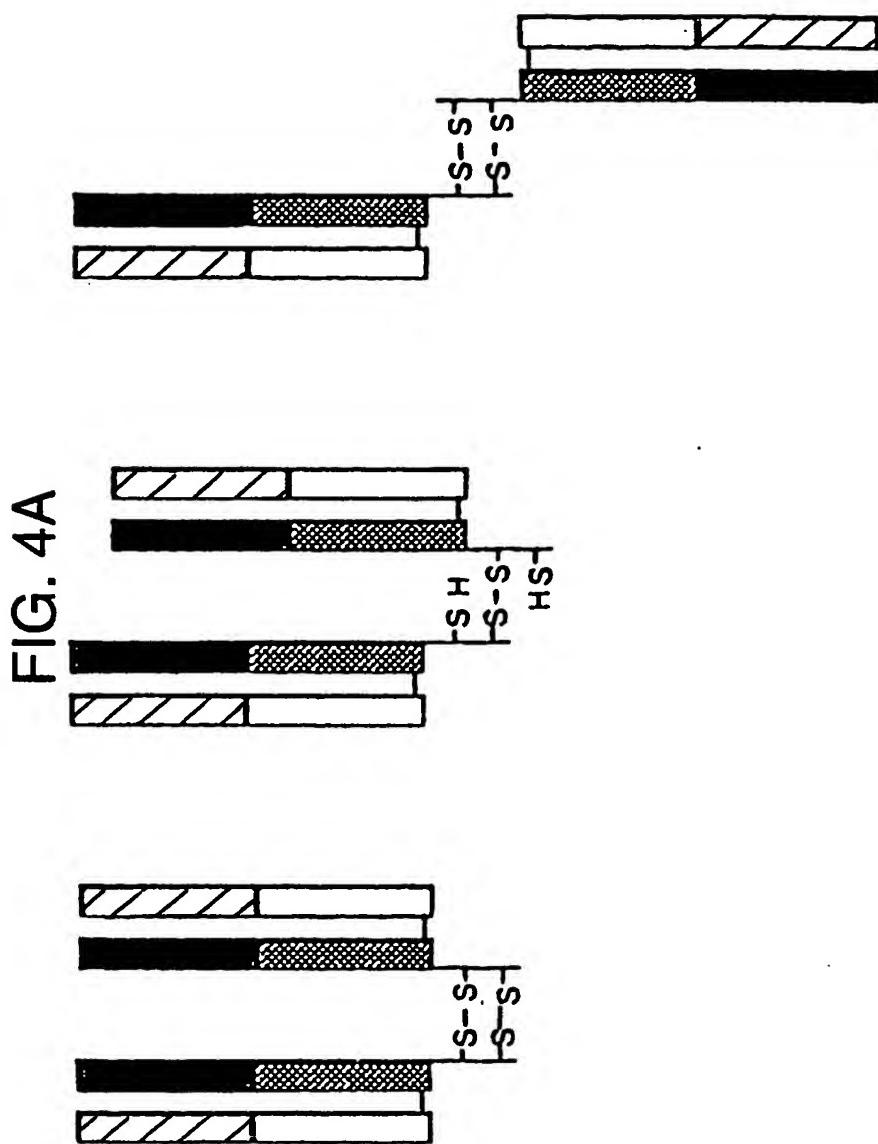


FIG. 3





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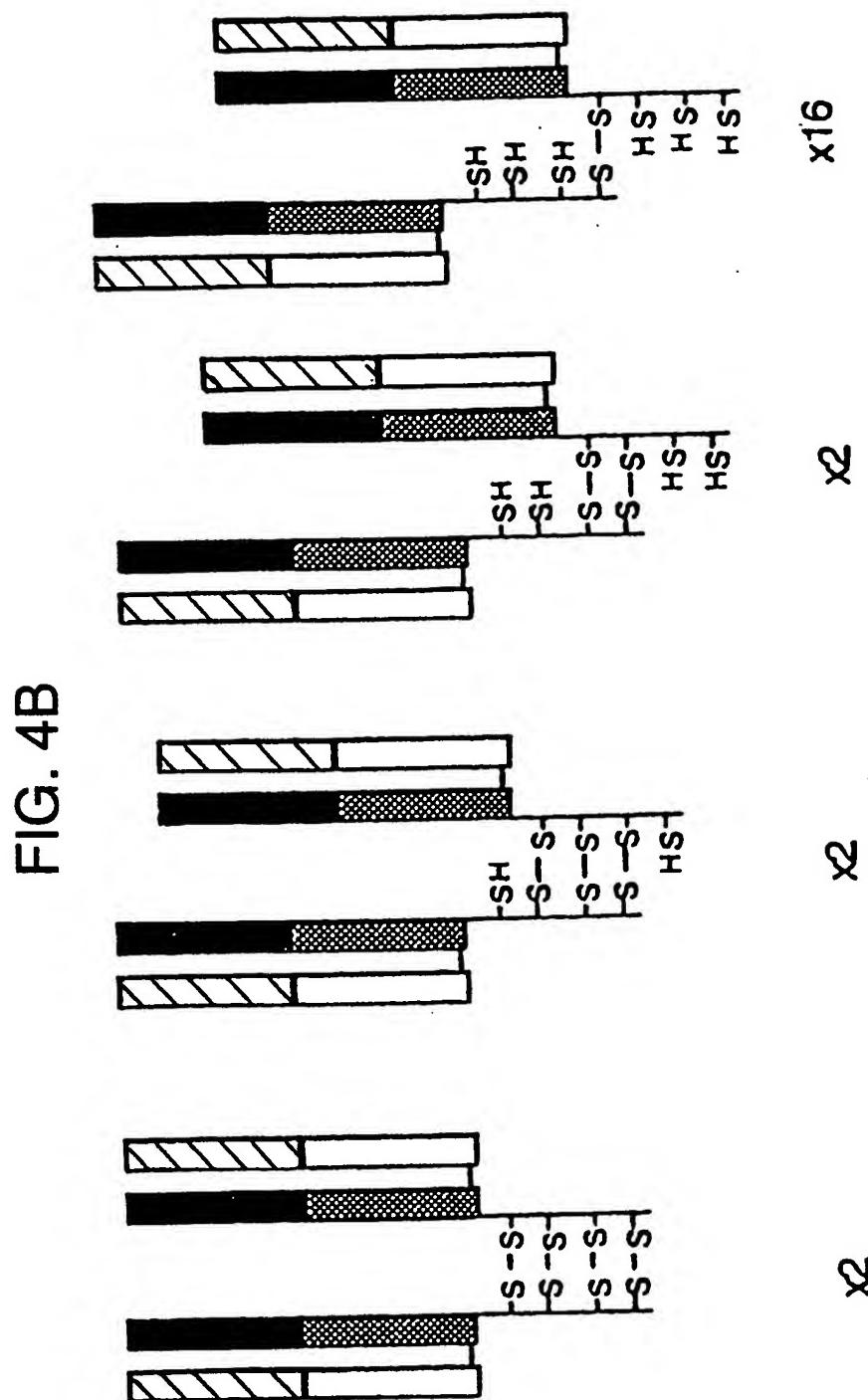


FIG. 4B

FIG. 5

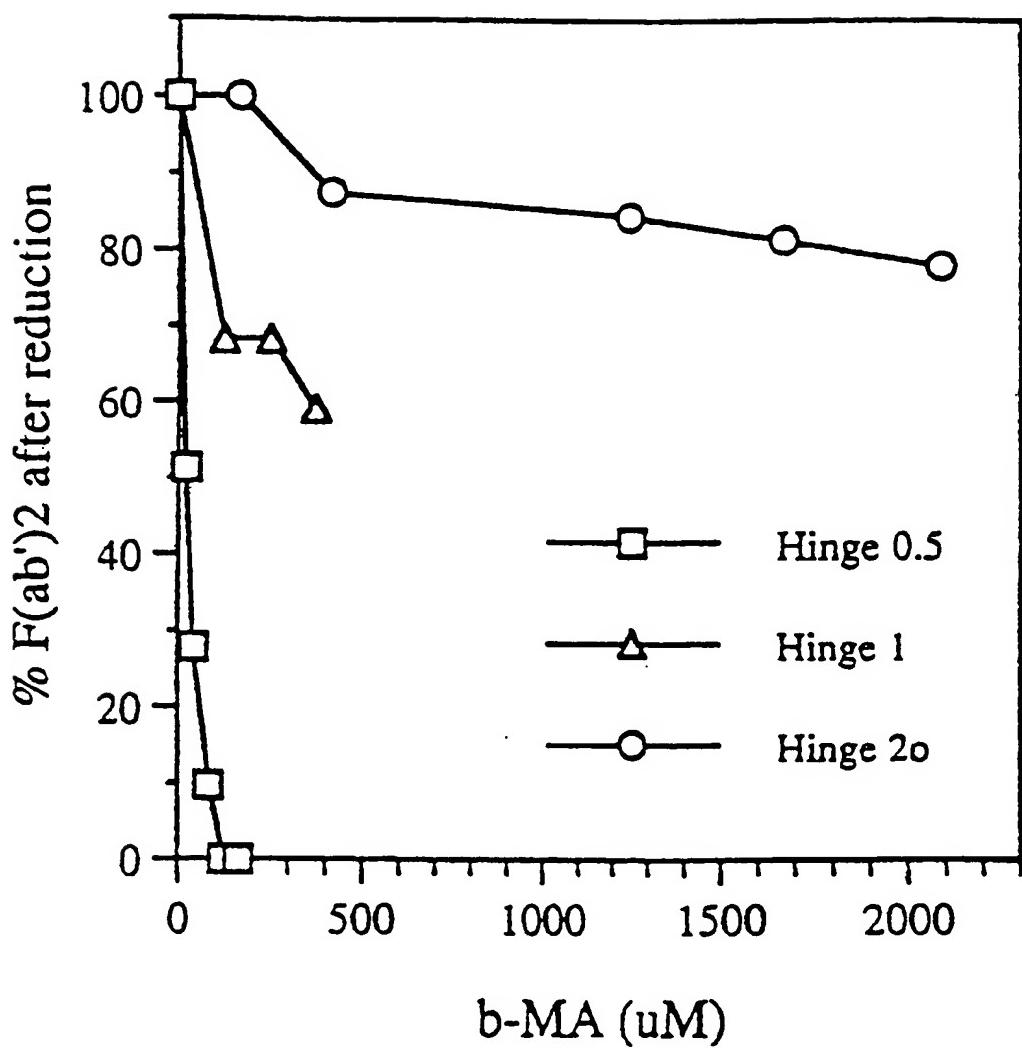
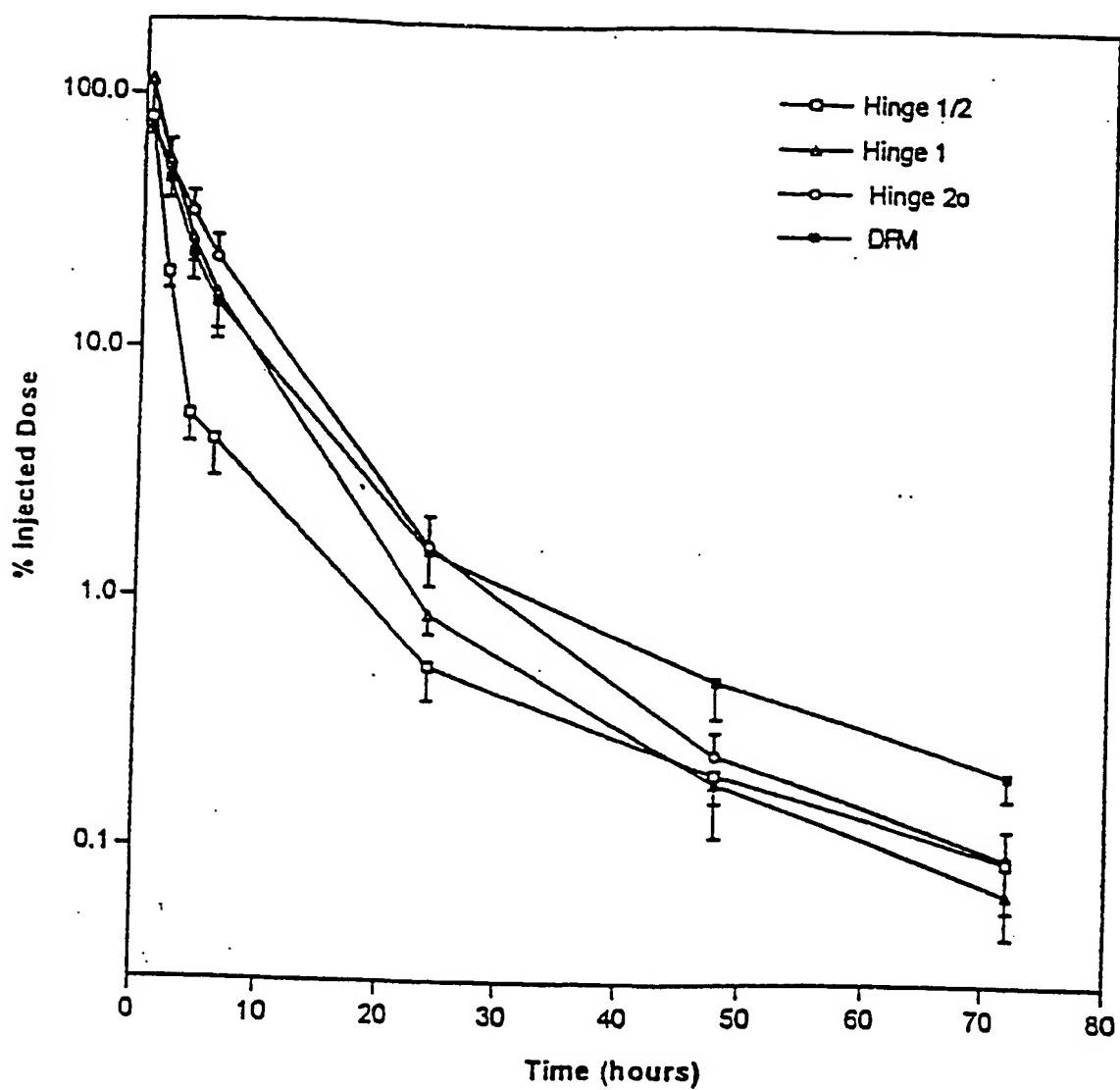


FIG. 6



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